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Degree Course Life Technologies

Option Biotechnology

Diploma 2010

Adel Hama

Enhancing product quantity by controlling the specific growth rate of *Pichia pastoris* expressing human serum albumin

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Mandant / *Auftraggeber* ☐ HES—SO Valais ☐ Industrie ☒ Etablissement partenaire **Dublin City University**	Etudiant / *Student* **Adel Hama**	Lieu d'exécution / *Ausführungsort* ☐ HES—SO Valais ☐ Industrie ☒ Etablissement partenaire
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Travail confidentiel / *vertrauliche Arbeit* ☐ oui / ja [1] ☒ non / nein		

Titre / *Titel*

Enhancing product quantity by controlling the specific growth rate of *Pichia pastoris* expressing human serum albumin (hSA)

Description et Objectifs / *Beschreibung und Ziele*

According to the FDA's Initiative for Industrial PAT, monitoring and control of a bioprocess has two principle aims:

- gather a deeper understanding of the on-going process and its underlying phenomena
- ensure a maximum productivity and an optimal quality of the desired product

The overall aim the 6-month Bachelor Work project should be to achieve the following objectives:

- Fed-batch cultures of a *Pichia pastoris* strain producing a recombinant protein (hSA) highlighting the relation between specific growth rate and product quantity and quality
  - Set up off- and on-line methods to follow the production of the desired recombinant protein (spectroscopy, HPLC, protein detection)
  - Perform a series of fed-batches applying the exponential feed strategies already in use in the LiB, with different set-points to maintain a desired specific growth rate
  - Analyze the data correlating the specific growth rate to product quantity and quality
  - Carry out a brief literature review about methods for testing the quality of the produced protein

The deliverables of this project are:

- A fully set-up, tested and optimised fed-batch strategy for the culture of *Pichia pastoris* producing hSA
- A detailed Bachelor Work thesis including a description of the optimised fed-batch strategy, results as well as background information about on-line monitoring and control of yeast fed-batch cultures and protein detection
- A presentation of the work at the end of the project

[1] *Par sa signature, l'étudiant s'engage à respecter strictement le caractère confidentiel du travail de diplôme qui lui est confié et des informations mises à sa disposition; il s'engage également à appliquer formellement la directive y relative.*

Durch seine Unterschrift verpflichtet sich der Student, die Richtlinie einzuhalten sowie die Vertraulichkeit der Diplomarbeit und der dafür zur Verfügung gestellten Informationen zu wahren.

Rapport reçu le / *Schlussbericht erhalten am* …….……. Visa du secrétariat / *Visum des Sekretariats* ……..

Délais / *Termine*

Attribution du thème / *Ausgabe des Auftrags:*
03.05.2010

Exposition publique / *Ausstellung Diplomarbeiten:*
—

Fin des travaux en laboratoires / *Ende Laborarbeiten:*
15.10.2010

Défense orale / *Mündliche Verteidigung:*
dès la semaine 40 / *ab Woche 40*

Remise du rapport / *Abgabe des Schlussberichts:*
25.10.2010 | 12h00

Signature ou visa / *Unterschrift oder Visum*

Responsable de la filière
Leiter des Studiengangs: .....

Etudiant/*Student*: .....

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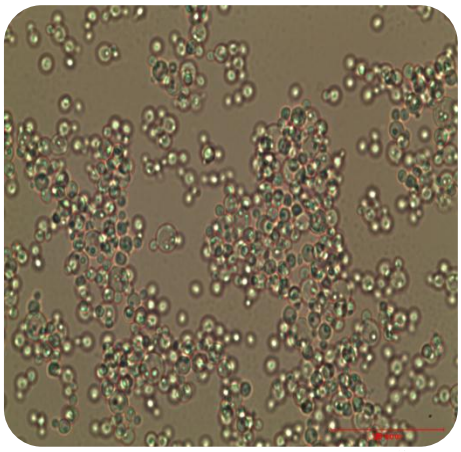
Enhancing product quantity by controlling the specific growth rate of *Pichia pastoris* expressing human serum albumin (hSA)

Graduate Adel Hama



Bachelor's Thesis
| 2010 |

Degree course
Life Technologies

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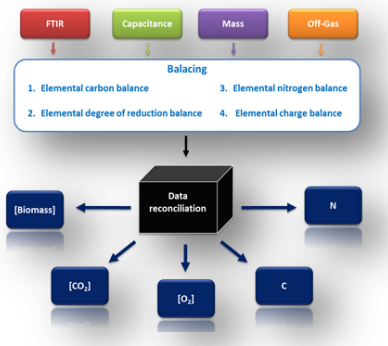
Objectives

Establish a fed-batch process to culture a *Pichia pastoris* strain producing a recombinant protein (hSA), highlighting the relationship between the specific growth rate and the protein quality, as well as seeking for enhanced productivity.

Methods | Experiences | Results

Pichia pastoris GS115/Mut^S/his-/sechSA is a methylotrophic yeast producing hSA using the alcohol oxidase promoter. Control of the specific growth rate (μ) is very crucial. The estimation of μ, biomass, off-gas composition and metabolites concentrations are done, in the frame of this work through data reconciliation based on elemental and mass balances. However, this is subject to influences by drifts in the devices and variation in the culture conditions. The fed-batch cultures are performed with glycerol as a carbon source. The batch contains 40 [g/L] glycerol and final biomass is 30 [g/L], $Y_{X/S}$ = 0.55 [g/g] and μ_{max} = 0.17 [h^{-1}]. Using an exponential feed profile, 0.5 L glycerol feed (500 [g/L]) is feeding at μ_{sp} and final biomass is 76 [g/L], $Y_{X/S}$= 0.40 [g/g]. Induction phase by methanol during 145 [h], the specific volumetric productivity is 6.6 [mg hSA/L/h]. We have shown the strain was not auxotrophic for histidine and therefore was potentially not carrying the hSA gene.

We also show the detection and quantification of BSA, hSA, Pepsin, Lipase and α-Amylase in water are possible with R^2 = 0.999. This result suggests on–line monitoring of protein are promising using FTIR.



Principe of data reconciliation: FTIR, capacitance, mass base and off-gas composition send data raw at MatLab. MatLab give new values more accuracy.

Wavenumber [cm⁻¹]	hSA [g/L] 0.156	0.313	0.625	1.25	2.5	5	10
1557	R²=0.9951						
		R²=0.9980					
			R²=0.9999				
				R²=0.9999			
1452	R²=0.9934						
		R²=0.9931					
			R²=0.9975				
				R²=1.0000			
1411	R²=0.9965						
		R²=0.9931					
			R²=0.9975				
				R²=0.9998			

Table show coefficients of determination for a range of 0.156-10 [g/L] of hSA in water.

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1. Introduction

Biotechnology is a scientific discipline with a very diverse field of application. Initially practiced for intellectual curiosity, over the years biotechnology has gained many industrial and commercial applications [1]. We find themes like molecular biology, genetics, bioinformatics, bioprocessing, etc… Nowadays, the economic stakes are very high, so governments invest a lot of money in research and development. Each year, many new medicines are commercialised in the several countries [2].

To ensure safety of the medicines going out on the health market, regulatory organisations are created in different countries. One of the most important is the US regulation authority the Food and Drug Administration (FDA). Due to the size of the US economic market. In order to obtain constant quality-oriented excellence, the FDA provides some guidelines that describe how to achieve process that will improve the quality of a product. In general, the FDA highly encourages the acceleration of innovation for the benefit of public health.

1.1.FDA's initiative: Process Analytical Technology (PAT)

According to the FDA, PAT is a system for design, analysis and control of manufacturing processes through timely and appropriate measurements to ensure the quality of final product [3]. Understanding of engineering, material sciences and quality assurance principles are crucial in the design of manufacturing processes to produce products of acceptable quality in a reproducible manner. The main objective is to improve the understanding of manufacturing processes and their control. Figure 1 shows the concepts that the FDA wants to consolidate for industrial companies and which is related to business excellence.

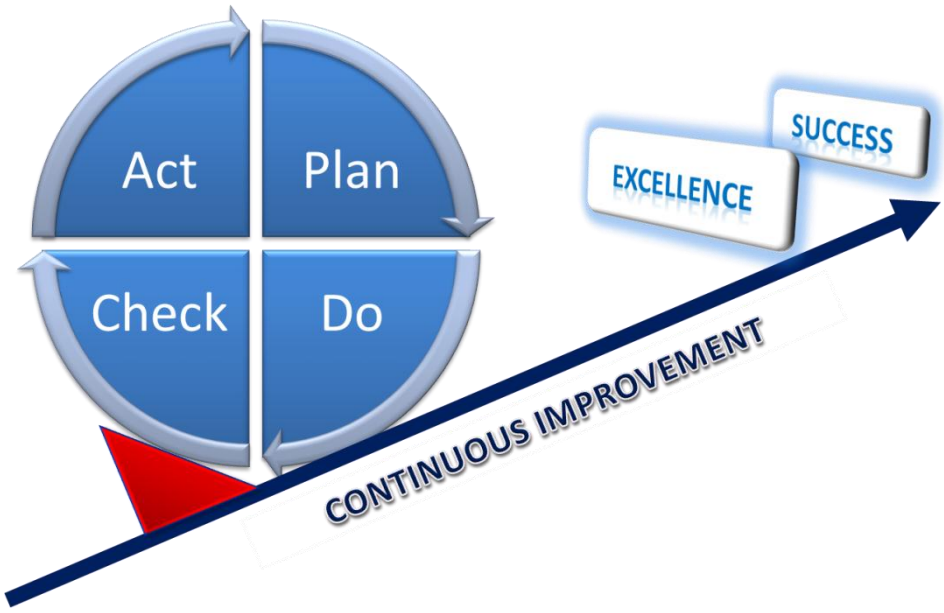


Figure 1: Schematic of the FDA's industrial concept

PAT uses many different tools for process understanding. Information gained makes optimisation and more precise process control possible which allows for risk minimisation and continuous process improvement. We can classify these tools according to certain criteria [3]:

- **Multivariate tools for design, data acquisition and analysis:** The final product is often derived from a combination of different processes so the number of variables is large. The development of programs allow the acquisition and consolidation of knowledge derived from process variables. Multiple variables are essential in the statistical design of experiments, methodology, etc... The development of theoretical models to simulate the process is very important in the continued search for knowledge and to reduce development time.

- **Process analysers:** Increased monitoring of what happens in the process is crucial. Knowing at a given moment the physical (Example: Temperature, oxygen, agitation, etc...) and chemical (Example: pH, substrate, protein, etc...) phenomena in the production line can aid control of the final product. Figure 2 shows four types of measurement taken :

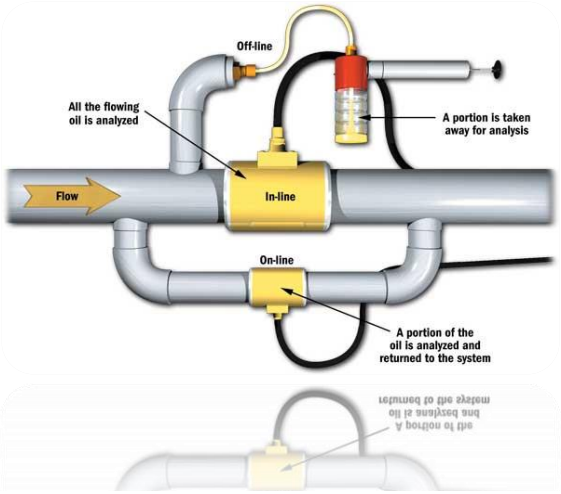


Figure 2 : General representation of measurement modes during manufacture. The picture shows three main ways: offline, online and in-line [4]

1. **Off-line :** The measured sample is extracted, isolated and analyzed off the production line.
2. **At-line :** The measured sample is extracted, isolated and analyzed close to the production line
3. **On-line :** The measured sample is analyzed and returned to the production line (Bypass loop)
4. **In-line :** The sample is measured directly in the production line, the measure can be invasive or non-invasive

In all cases, it is clear that the development of in-line measurement methods is crucial. Indeed, what happens in the production line with an almost instantaneous response time can minimize the deviations which may affect the final product.

- **Process control tools:** Strict control of all factors affecting the production of the product should be established. This control will significantly reduce risks and thus maintain the desired quality of product. Taking into account the characteristics of the equipment (machine, sensor, analyser, etc...) and raw materials is an essential prerequisite for the establishment of quality assurance.

- **Continuous improvement and knowledge management tools:** It is important to carefully record the knowledge gained during product development and during its manufacture as well as over the life cycle of the product does not end with its removal at the end of the process but with its use by the public. Such data is always useful and can validate changes to made to processes. The resulting information can consolidate the knowledge and databases already in existance and also open up new possibilities in the manufacture of similar compounds. Having a view to improving process knowledge and hence manufacturing is a term referred to excellence.

The implementation of PAT is highly recommended by the FDA. Bioprocesses have been used for thousands of years such as the use of microorganisms for production of alcohol, bread, cheese, etc... This discipline has continued to expand and modernise. Controlling the growth of these microorganisms can significantly influence the production of compounds in terms of quantity and quality. In this study, the fermentation of yeast, *Pichia pastoris*, will be carried out to produce the protein human serum albumin (hSA). In agreement with the FDA guidelines, an analysis of data will be established based on off-line and on-line measurements of the cultivation process in order to increase productivity. **The ultimate goal is to ensure/control maximal production level and optimal quality of the hSA by controlling, as finely as possible, the specific growth rate of strain.**

1.2.Bioprocess monitoring and control

The FDA initiative, PAT, is essentially based on voluntary participation of companies and laboratories. As mentioned in the previous section, knowing the physical and chemical phenomena that occur in our bioprocess (fermentation) is fundamental. The next step is obviously the control. In fact, to optimise the fermentation and thus production of hSA, it is necessary to link the monitoring and control in the process. The ability to act according to the situation encountered depends on the data collected and the time needed to acquire them. Only measuring methods that were actually word are discussed.

1.2.1. Monitoring of fermentation

Like all microorganisms, growth is influenced by certain parameters such as pH, temperature, substrate, pO2, the amount of CO2, etc... Monitoring the evolution of these parameters allows visualisation of the hypothetical physiological state of yeast. The development of analytical methods and the choice of the method are key elements in achieving the objectives of the project. To validate the values collected on-line, a comparison is made between the results obtained by the off-line and on-line methods.

1.2.1.1. Off-line measurements

Dry Cell Weight

During fermentation, growth of yeast increases with time. The mass of dry yeast also increases. After centrifugation of samples collected, they are dried to remove residual water. This method is commonly used in laboratories, however, it is time consuming and when applied to fermentations using media conducive to the formation of solid particles, it may overvalue the value of biomass over time. But two major disadvantages of the method the lack of distinction between live and dead cells in biomass determination and the sensitivity of the method which is only 50 [mg/mL] [5].

Optical Density

This method is much more sensitive 0.1 [mg/mL] [6]. It is based on the property of the sample to absorb a beam of monochromatic light at 600 nm (Figure 3). The more cloudy a sample is, the greater the amount of cells there is and therefore less light is absorbed. Thus one can easily correlate the number of cells with the absorbance versus time. However, it also does not discriminate between the livings and dead cells indiscriminately or the media turbidity but the volume necessary is smaller.

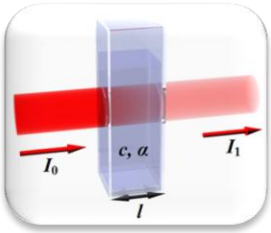
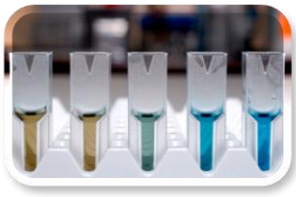


Figure 3 : Schematic representation of the Beer Lambert law $A = \varepsilon.l.C = -\log_{10}(I_1/I_0)$ [7]

Determination of Protein



In laboratories, the Bradford method is used to determine the amount of protein in solution in the fermentation medium or after cell lyses. It is a colorimetric method where the increase in absorbance at 595 nm is proportional to the amount of protein in the reaction medium (Figure 4). The reference protein, to determine the concentration of protein is bovine serum albumin (BSA). This determination represents the total protein concentration in the sample. It does not differentiate between total protein, secreted proteins or proteins released due to cell death.

Figure 4 : Standard solutions of protein that reacts with the reagent of Bradford. The amount of protein increases from right to left [8]

Determination of Metabolite Concentration

Metabolic monitoring is crucial during fermentation. Method of high-pressure liquid chromatography (HPLC) can separate analytical and quantify several metabolites at the same time with great accuracy. It's a method based affinity of compounds for stationary or mobile phases. In this case we can analyze glycerol, ammonium chloride, ethanol and methanol concentration.

1.2.1.2. On-line Measurements

In industry, the desired mode for monitoring fermentations is online and in-situ. In order to carefully control the yeast growth, it is important to follow with the greatest possible precision the various parameters for the fermentation. The monitoring of physical phenomena as well as their influence on the metabolism, will improve knowledge of the fermentation process, and help to establish corrective actions to optimize and achieve production goals.

Temperature, pH, pO_2, Agitation

Maintaining certain parameters such as temperature, pH and pO_2 constant, is crucial to maintain the desired physiological state of the organism. For example, the change in pH can significantly influence the three-dimensional conformation of the enzymes needed for growth but also the synthesis of the product of interest. The temperature will affect different phenomena (medium viscosity, density, substrate transfer, oxygen transfer, etc...). It is obvious that the oxygen must be rigorously monitored and controlled. The method of fermentation of the cell will be influenced by the amount of oxygen dissolved in the media and thus reaching the cell. At steady state, the oxygen transfer rate (OTR) is an important value thereby determining the amount of oxygen consumed (OUR). This parameter becomes essential to establish a high cell density culture [7]. Small amounts of dissolved oxygen induce the cell to a fermentative mode of cultivation while a sufficient intake induces an oxidative mode. However, pO_2 is not the only parameter that determines the mode of culture during fermentation.

Analyse Gas Out

During fermentation, gas is produced and consumed. Consumption is induced by the oxygen supply while production is the release of carbon dioxide by yeast. An important variable in yeast fermentation is the respiratory quotient (RQ). It is the ratio between changes in the rate of carbon dioxide (CER) and the rate of oxygen consumption (OUR). A fermentative metabolic pathway will have a RQ greater than 1 and the oxidative process will have an RQ between 0.9 - 1 [9]. The gas analysis is crucial; it will monitor the behaviour and evolution of yeast under certain fermentation conditions. This analysis helps to understand and make assumptions about what is happening in the cell (black box). To establish mass balances, it is important to control the composition of gas entering and exiting the reactor.

Dielectric Spectroscopy

More known as capacitance, dielectric spectroscopy can measure the biomass in a reactor. It is a reliable technique, which is widely used in laboratories. The surface of the membranes are polarised under the action of an electric field induced by the probe (Figure 5). A capacitance is created and after extinguishing the electric field. When the yeast grows, there is increased capacitance. There are more cells formed so that more surfaces are polarised. We can easily correlate the total number of cells with capacitance during fermentation. The detection limit was 10^5 [cells/mL] with a measuring frequency of 2 min^{-1} [11]. In this study the detection limit is 10^6 [cells/mL] (*Saccharomyces cerevisiae* - 6 µm) or 0.5 [g/L] with a resolution of 0.1 [pF/cm] [12]. Its great advantage is that it only takes into account the viable cells and is not significantly affected by cellular debris, the compounds of the environment or even the variation of pH [13].The material used in this study has a resolution of 0.1 [pF/cm] and detection of 10^6 [yeast/mL] (0.5 [g/L]) [14].

Figure 5 : Schematisation action the probe on cells [10]

Fourier Transform Infrared Spectroscopy (FTIR)

This method of analysis is increasingly studied in bioprocessing. Initially, infrared spectroscopy (IR) was used routinely in quality control laboratories to verify the identity and purity of raw material. It has since been developed and has become an instrument for on-line non-invasive analysis of several compounds [15-16]. This technique is based on the principle of energy absorption of molecules at specific wavelengths. Energy in form of an electromagnetic wave induces a molecular vibration. If the resonant frequency of the bond is the same and there is a change in the dipole moment, we observe a peak in the IR. Unlike the dispersive type of infrared (IR), FTIR allows analysis of several compounds simultaneously. It gives a spectrum of all frequencies of light source by using the interferometer. Generally the range of wavelengths in the mid infrared is 2.5-25 µm corresponding to 4000-650 cm^{-1}. This very innovative method has significant advantages compared to the dispersive system: fast measurement (1 second against 30-minute dispersive), high signal/noise ratio, precision, high sensitivity and stability [17-19]. With these characteristics, FTIR has all the potential to monitor in-line a bioprocess. Moreover, the presence of cells does not affect measurements and is non-invasive [20].

Data Reconciliation

The application of spectroscopic methods has inherent problems including instrumental drift and process drift during the calibration. To overcome this problem, the method of data reconciliation can correct the estimated concentrations collected by analytical instruments [21]. It is defined as a statistical technique that evaluates the consistency of measures taken and reduction of random errors by taking into account constraints such as equalities and/or physical inequalities [21]. Figure 6 shows how the reconciliation of data is used in this work.

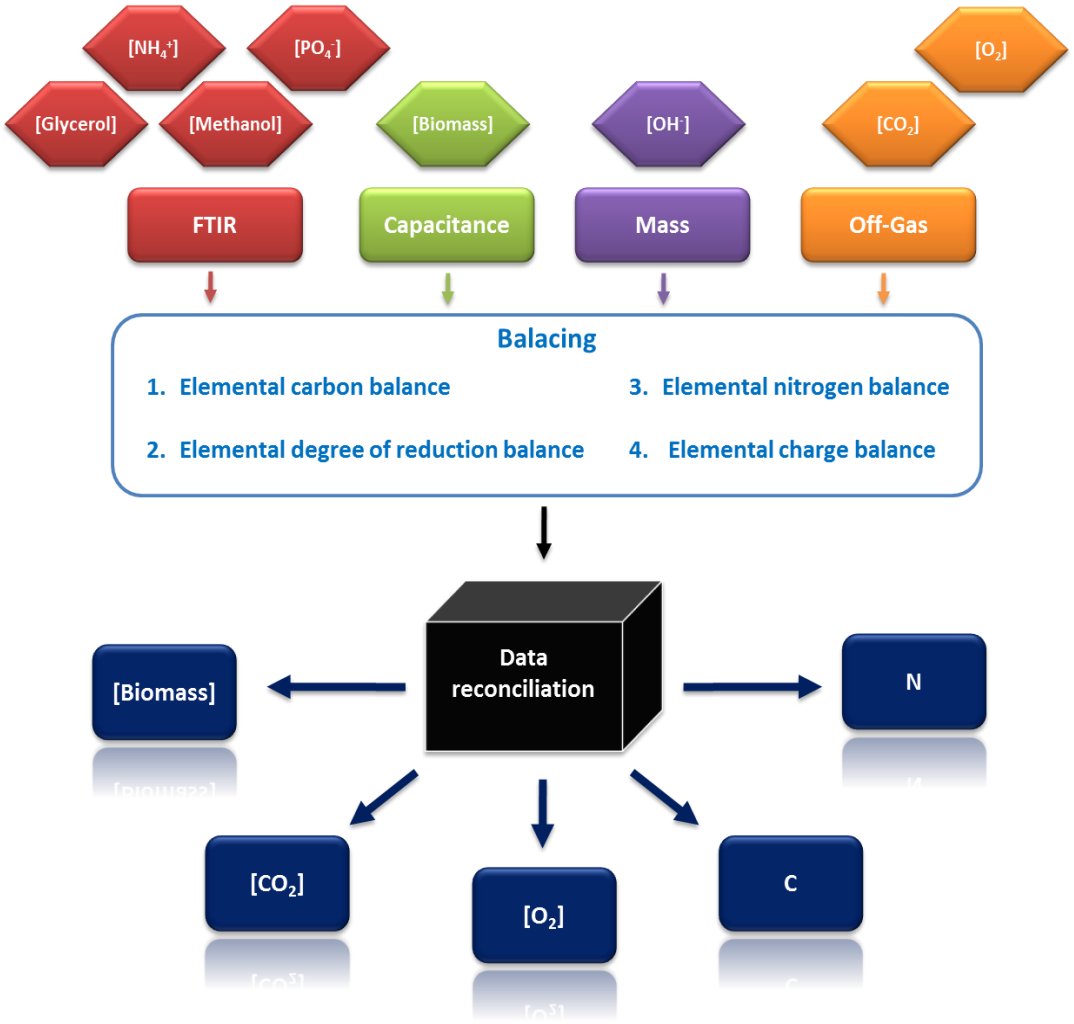


Figure 6: Principle of data reconciled during process

1.2.1.3. Estimation specific growth rate μ

The specific growth rate is a key element during fermentation. It is influenced by culture conditions but more importantly it depends on the strain used. During the development of bioprocesses, the emphasis is placed heavily on estimation and control of this parameter. The growth of yeast follows an exponential growth with specific growth rate comes into at stake [20, 22], whichever equation1.

$$X_t(t) = X_{t=0} \cdot e^{\mu.t}$$

Equation 1 : Calculation for the biomass concentration X_t [g/L] generate at time t from the biomass initial concentration $X_{t=0}$ [g/L] with the parameter the specific growth rate μ [h^{-1}]

To produce a product quickly and at high levels, it is clear that we must have a high biomass concentration. In this sense, obtaining a high density cell culture becomes the goal, in order to produce the product of interest at high concentrations. To do this, methods must be established which will estimate as precisely as possible the specific growth rate and therefore help to control cell growth. Equation 2 is used to estimate the specific growth rate as follows.

$$\mu_{est}(t) = \frac{ln(\frac{X_t}{X_{t-1}})}{t_t - t_{t-1}}$$

Equation 2 Estimation for the specific growth rate μ_{est} [h^{-1}] at time t [h] where X_t [g/L] is the biomass concentration at time t [h] and X_{t-1} [g/L] is the biomass concentration at time t-1 [h]

The instability of instruments (agitation, noise, temperature, etc...) is a problem affecting the measurement and thus the value of μ_{est}. For this, the specific growth rate was estimated at a regular time interval of 20 minutes, thereby lessening the impact of drift on the value μ_{est} [23].

1.2.2. Control of fermentation

As discussed in the previous section, control of fermentation is an important tool to achieve the objectives. It is a sizeable challenge because it is difficult to precisely control cell metabolism because that several variables can influence the system. Indeed during the fermentation, the cell metabolism will adapt its metabolism to environmental conditions. They will also change during the fermentation [23]. The growth is exponential, the controllers must adapt over time and to express the exponential function. Also following the exponential function, the error will increase. An overview of fermentation control used in this work is given in figure 7.

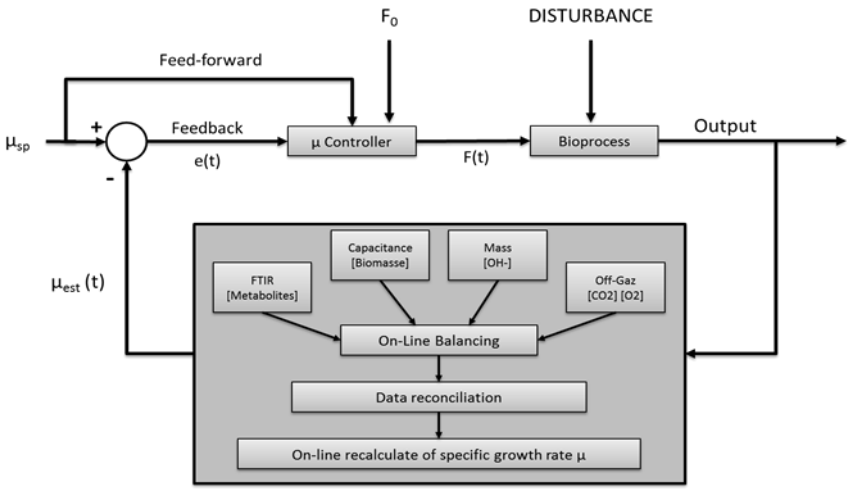


Figure 7: Functional diagram of the system

To minimize variation of error and maximise response, we use a proportional-integral controller (PI). The principle of the controller is a simple feed-forward/feedback. In view of the FDA, this approach is very interesting as it allows a simple approach in the regulation of fermentation. The integration of proportional and integral terms in the exponential function describing the growth will contribute to the robustness of the controller [23].

The control is governed by **equation 3** below. It takes into account the balance of the initial substrate and biomass.

$$F_{FF}(t) = F_0 \cdot e^{\mu_{sp} \cdot t} \text{ with } F_0 = X_0 \cdot V_0 (\mu_{sp} / (Y_{X/S} \cdot S_F))$$

Equation 3 : Calculation of the feed-forward rate controller F_{FF} [L/h] at time t [h] Where F_0 is the initial rate [L/h], μ_{sp} is the specific growth rate set point [h^{-1}], X_0 is the biomass initial concentration [g/L], V_0 is the initial volume in the reactor [L], $Y_{X/S}$ is the biomass yield coefficient [-] and S_F is the substrate concentration in the feed solution [g/L]

At each time interval, the difference between the set point μ_{sp} and the estimated μ_{est} value is calculated according to **equation 4**.

$$\varepsilon(t) = \mu_{sp} - \mu_{est}(t)$$

Equation 4 : Expression calculating the error term by difference the specific growth rate μ_{sp} [h^{-1}] and the specific growth rate μ_{est} [h^{-1}] at time t [h]

By integrating the error term $\varepsilon(t)$ and a proportional term K_p, **equation 3** yields **equation 5**. This will compensate for any variation from the error term F_0.

$$F(t) = F_0 \cdot e(\mu_{sp} + K_p \cdot \varepsilon(t))t)$$

Equation 5 : Calculation for the feed-forward rate proportional controller F_{FF} [L/h] integrating the proportional term K_p [-] to error term $\varepsilon(t)$ [h^{-1}] at function time t [h]

The advantage of control, via the proportional term is that it uses only the error as a parameter. However because of this non-selectivity in the amplification gain, the steady-state error becomes significant and non-zero at equilibrium **[23-24]**. Thus we must increment **equation 5** with a new term that will strengthen and stabilise the regulator. This term is integral, therefore the coupling proportional and integral terms will significantly reduce the effect of the static error.

$$F(t) = F_0 \cdot e(\mu_{sp} + Kp \cdot \varepsilon(t) + Ki \cdot {}_0\int^t \varepsilon(t).dt\ )\ t)$$

Equation 6 : Expression calculating the feed-forward rate controller proportional-integral F_{FF} [L/h] integrating the proportional term K_p [-] and the integral term K_i [-] to error term $\varepsilon(t)$ [h^{-1}] at function time t [h]

This type of controller is called PI controller. However, this kind of controller has an oscillatory behaviour and there is instability linked to the excessive increase in the integral gain. Thus the value of K_p and K_i terms are key factors in the control. In this sense, the optimisation of these values is a critical step especially since they must be sufficiently robust to compensate for possible disruptions of specific growth rates **[23]**.

1.3. *Organism model Pichia pastoris GS115*

Pichia pastoris GS115 is a methylotrophic yeast strain widely used in the production of recombinant protein. It is capable of performing the process of glycosylation but is not 100% compatible with humans. As a result of these properties, it is mainly used to reach high cell density in cultures (HCDC: High Density Culture Cells) **[25]**. Moreover, *Pichia pastoris* is used in this study because it is Crabtree-negative. This feature helps to inhibit the bottleneck effect (overflow metabolism) leading to the production of ethanol. However in the absence of oxygen, the metabolism becomes fermentative and ethanol is produced. This strain allows the expression of secreted proteins thanks to its metabolism. **Table 1** show various proteins which can be produced by this strain including the hSA between 1 and 3.4 [g/L].

Table 1 : Heterologous proteins synthesized in fermenters [25]

Protein	Amount secreted and signal sequence used	Fermenter volume (L)	Culture density
Atrazine-specific Fab fragment K411B	S, 40 mg/L, α-MF	4	NS
Chrondrus crispus hexose oxidase	S, 250 mg/L	NS	NS
Heavy-chain fragment C of botulinum neurotoxin	I, 219 mg/kg	60	165 OD_{600} units/ml
Human antithrombin III	S, 15 mg/L, *SUC2*	2.7	NS
Human bile salt stimulated lipase	S, 800–1000 mg/L, *SUC2*	3.5	NS
Human chitinase	S, 300 mg/L, α-MF	1.5	400 g/L (wet cell weight)
	S, 1.5 g/L, α-MF	15	600 OD_{600} units/ml
Human cystatin-C	S, 650 mg/L, α-MF	2	1.2 g/L (dry cell weight)
Human insulin	S, 1.5 g/L, α-MF	16	>500 OD_{600} units/ml
Human pS2/trefoil factor 1	S, 20 mg/L, α-MF	10	22 OD_{600} units/ml
Human serum albumin	S, 1–3.4 g/L, native	8.5	101 g/L (dry cell weight)
Human type I collagen	S, 500 mg/L, α-MF	1	NS
Malaria vaccine antigen, $P30PsMSP1_{19}$	S, 500 mg/L, α-MF	2.5	180 g/L (wet cell weight)
Synthetic gelatin	S, 3–6 g/L, α-MF	NS	NS

S, secreted; I, expressed intercellularly; NS; not specified; α-MF, yeast α mating factor signal sequence; *SUC2*, yeast invertase gene..

1.3.1. Expression system Mut^S

It is very important to be able to control what time you want to get the product of interest, i.e. the mode of expression. There are two types of expression system commonly used in laboratories for strain *Pichia pastoris* [25]:

- **Mut$^+$** (methanol utilization high): The gene expressing the recombinant protein of interest is inserted into the genome of yeast. It is located adjacent to the gene expression of alcohol oxidase (AOX1 gene) and under the control of AOX1 promoter. The induction is performed by methanol and repressed by glycerol. The strain with this expression system can be grown effectively with the methanol as an energy source.

- **Mut^S** (methanol utilization slow): Here the gene encoding the recombinant protein is inserted into the genome of the yeast in the AOX1 gene and under the control of AOX1 promoter. The induction is done by methanol and repression by glycerol. The strain has a low growth with methanol as an energy source and only if the protein is expressed AOX2. Growth will be strong with glycerol as an energy source.

In this practical work, the strain used is *Pichia pastoris GS115/Mut^S/his-/sechSA*. The expression system used, Mut^S, and allows production of the protein hSA only when the fermentation medium is devoid of glycerol and contains methanol. In addition, the Mut^S strain produces more protein than the Mut$^+$ strain [26]. The hSA will be secreted into the medium and a solid/liquid separation will isolate the protein of interest in biomass.

1.3.2. Induction by methanol

The production of hSA is performed by the signal caused by the interaction of methanol on the AOX1 promoter (in the absence of glycerol as repressor). To have high hSA productivity, it is essential to obtain high cell density. When the desired HCDC is obtained, the induction may take place, however, methanol can cause cell death because of its toxicity at high concentrations. Thus the change of substrate, glycerol-methanol, must be done carefully and in time for a proper adaptation to the new substrate [26].

Thus, in this practical work based on the cultivation of *Pichia pastoris*, we performed a fed-batch cultures of a *Pichia pastoris* strain producing a recombinant protein (hSA) highlighting the relationship between specific growth rate, product quantity and quality the following procedure:

- Set-up off-line and on-line methods to follow the production of the desired recombinant protein (spectroscopy, HPLC, protein detection).
- Perform a series of fed-batches applying the exponential feed strategies already in use in the LiB, with different set-points to maintain a desired specific growth rate.
- Analyse the data correlating the specific growth rate to product quantity and quality.
- Carry out a brief literature review about methods for testing the quality of the produced protein.

The output of the work is to set-up a fed-batch culture of *Pichia pastoris* producing hSA with the optimized the process conditions.

2. Material and methods

2.1.Cell Strain

Pichia pastoris GS115/Mut^S/his-/sechSA, a Crabtree-negative yeast was obtained from Laboratory of Integrated Bioprocessing, School of Biotechnology, Dublin City University, Dublin, Ireland. The strain for the master cell bank (MCB) was stored at -80°C in 1.8 mL YPGlycerol medium (6 [g/L] Yeast extract, 5 [g/L] Peptone, 20 [g/L] Glycerol).

To create the Working Cell Bank, sterilize 1 L of YPGlycerol. 1.8 mL of a previous working cell bank (WCB) were added to 100 mL of sterile YPGlycerol in a 1 L shakeflask under a laminar flow (TC 48, Gelaire® Flow Laboratories) and grown at 30°C, 150 rpm for 24 h in a shaker incubator (SL Shel Lab, SI SERIES, SHELDON MFG Inc.). 30 ml of this culture were centrifuged for 10 minutes at 4000 rpm at 4°C (FL40R Centrifuge, Thermo Scientific) and the supernatant was removed. The pellet was suspended in 6 mL sterile 0.9 % NaCl and 6 mL sterile 0.2 % Glycerol solution. The suspension was aliquoted in form of 1.8 ml in sterile 2 ml tubes and frozen at -80°C (Ultra-Low Temperature Freezer, MDF-73865, Sanyo Electric Co., Ltd).

Product	Reference
Yeast extract	Y1333, Melford, Sulffock
Peptone	P/1160/50, Fisher scientific, UK
Glycerol	Glycerol 99%, 15523, Sigma-Aldrich, Germany
NaCl	71379, Fluka, Sigma-Aldrich, Germany
Balance	Mettler AE 163, Mettler Toledo, Suisse
Autoclave	Tline™© Exor R&D, Fedegari Autoclavispa, Italia

2.2.Culture media

2.2.1. Pre-culture and inoculums preparation

A 500 mL shakeflask, 100 mL of BSM medium (See section 2.2.2 Media) at pH 5 was inoculated with 1.8 mL of WCB *Pichia pastoris GS115*. Cultivation took place in an incubator (SL Shel Lab, SI SERIES, SHELDON MFG Inc.) set to 30°C and 200 rpm for 24 hours. Twice 45 ml of the culture was distributed in 2 sterile 50 mL tubes and centrifuges for 10 min at 4000 rpm at 4°C (FL40R Centrifuge, Thermo Scientific). The supernatant was discarded and the pellet suspended in 10 mL 9 [g/L] NaCl solution. This pre-culture was used to inoculate the bioreactor.

2.2.2. Media

For the batch, a simple basal salt medium (BSM) was used in a 3.6 L bioreactor (Bioengineering AG, Wald, Switzerland). 2 L of medium was prepared for the batch phase. The medium contains per litre: 13.35 mL 85 % H_3PO_4, 0.59 g $CaSO_4 \cdot 2H_2O$, 9.10 g K_2SO_4, 7.45 g $MgSO_4 \cdot 7H_2O$, 2.06 g KOH, 40 g Glycerol, 9.00 g NH_4Cl. 4 M NaOH was used to adjust the pH to 5 before adjusting the volume. The medium was filtered with a 0.22 µm sterile filter (GP Millipore Express® Plus Membrane, 250 mL Funnel, 45 mm Neck Size, SCGPT02RE, Millipore Corporation, USA). 4.35 mL sterile PTM1 trace element solution and 10 mL 0.4% L-Histidine stock solution (L-Histidine, 53319-25G, Sigma, Japan) were added to each litre of batch solution. These solutions were filtered with sterile 0.22 µm filter (GP Millipore Express® Plus Membrane, 250 mL Funnel, 45 mm Neck Size, SCGPT02RE, Millipore Corporation, USA). The PTM1 solution contained 6.00 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.00 g $MnSO_4 \cdot H_2O$, 0.20 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_3, 0.92 g $CoCl_2 \cdot 6H_2O$, 20.00 g $ZnCl_2$, 65.00 g $FeSO_4 \cdot 7H_2O$, 0.20 g Biotin and 5.00 ml of concentrated H_2SO_4 per litre. The solution of antifoam PPG200 was added manually to control the foaming in the bioreactor. The glycerol feed solution contained 500 g glycerol, 45 g NH_4Cl and 12 mL PTM1 per litre. It was sterilised by filtration (GP Millipore Express® Plus Membrane, 250 mL Funnel, 45 mm Neck Size, SCGPT02RE, Millipore Corporation, USA). The methanol feed solution contained 1000 g methanol and 12 mL PTM1 per litre.

This preparation was based on a recipe for *Pichia* fermentation medium by Invitrogen [27].

Product	Reference
H_3PO_4	Ortho-Phosphoricacid 99%, 79622, Fluka, Suisse
$CaSO_4$, $2H_2O$	Calcium Sulfate Dihydrate min 99%, C3771-500G, Sigma-Aldrich®, Japan
K_2SO_4	Potassium Sulfate, SigmaUltra, min 99%, P9458-250G, Sigma-Aldrich®, Japan
$MgSO_4$, $7H_2O$	Magnesium Sulfate Heptahydrate Puris, 13142, Riedel-deHaïn, Germany
KOH	Potassium Hydroxyde, P5958-500G, Sigma-Aldrich®, Sweden
Glycerol	Glycerol 99%, 15523, Sigma-Aldrich, Germany
NH_4Cl	Ammonium Chloride puriss, 11209, Riedel-deHaïn, Germany
$CuSO_4$ $5H_2O$	Copper (II) Sulfate Pentahydrate, cryst. Extra pure, 1.02787, Merck, Germany
NaI	Sodium Iodide, S-8379, Sigma®, USA
$MnSO_4$ $7H_2O$	Magnesium Sulfate-7-Hydrate, 291175X, BDH, England
Na_2MoO_4 $2H_2O$	Sodium Molybdate Dihydrate, 1.06521.0250, Merck, Germany
H_3BO_3	Boric Acid cryst. Extra pure, 160.1000, Merck, Germany
$CoCl_2$ $6H_2O$	Cobalt(II) Chloride Hexahydrate, 98%, A.C.S. reagent, 25,559-9, Aldrich, Germany
$ZnCl_2$	Zinc chloride, 98%, A.C.S. reagent, 21,127-3, Sigma-Aldrich®, Germany
$FeSO_4$ $7H_2O$	Ferrous Sulfate Heptahydrate, F8048-250G, Sigma®, USA
Biotin	Biotin, ≈99%, (TLC), B4501-1G, Sigma-Aldrich®, USA
H_2SO_4	Sulfuric acid 95-97%, 1.00731.2511, Merck, Germany
PPG200	PolyPropylene Glycol P 2'000, 81380, Fluka, Belguim
Methanol	Methanol CHROMASOLV®, 34860, Sigma-Aldrich®, Israel

2.3.Fermentation condition

Pichia pastoris fermentation was performed using 2 L working volume in 3.6 L bioreactor (Bioengineering AG, Wald, Switzerland) (Annexe 1). This reactor is equipped with a double 6-blade Rushton-type agitator, baffles, temperature and pH and capacitance probes. There are also control mechanisms, air inlet and air outlet ports, a base inlet port, a feed inlet port and sampling port. A condensation system allows the air outlet to cool and minimize liquid loss by evaporation. The pH is maintained constant at pH 5 with a solution of 4 M NaOH without acid control. The sterilization was executed *in-situ* with water at 121 °C for 20 min, the FTIR loop was also for 10 min. Then the reactor was drained when cooled to room temperature and filled with 2 L of sterile medium. The agitation speed was set to 800 rpm, the temperature was 30 °C and the inlet air flow rate was 2.5 L/min (1.25 vvm). During the culture, the agitation speed was increased to 1200 rpm for keep an oxygen transfer appropriate. The metabolites were monitored by FTIR (ReactIR™ 4000 FTIR, Mettler Toledo, Switzerland) and CO2 and O2 by agas analyser (Duet Dual Gas Sensor, AB Duet, Advanced Biosystems Ltd, UK) . Inoculum was injected manually using syringe including 1 mL antifoam. The first step was in batch mode until the cells consumed the entire carbon source and at that moment the glycerol feed was started to obtain a high cell density culture (HCDC). When HCD was reached, the methanol feed was started for the protein production.

During the fermentation, 12 mL of sample were taken at regular interval. They were used for the off-line analyses: Dry cell weight, metabolites concentrations by HPLC, optical density measurements at 600 nm and protein detection by Bradford assay.

The softwares required to configure the devices, as well as the devices themselves are describe in literature [21] (Annexe 2) and were namely Gas analyser, Biomass Monitor, FTIR, Matlab and Labview.

In the following subsections, each method used throughout this work will be quickly described.

2.4.Dry Cell Weight

In the first step, a 1 mL sample is taken and poured into a dried tube of known mass. The tube is centrifuged (Centrifuge 5415, Eppendorf AG, Germany) at 10'000 rpm and 4°C for 10 min. The supernatant is discarded and the tube is put in an incubator (Thermocenter TC-100S, Renggli AG, Switzerland) at 100°C overnight. The tube is weighted and the DCW [g/L] is calculated.

2.5.Determination of metabolite concentration

Glycerol, Ethanol, Methanol and Ammonium concentrations are determined using a HPLC method. 1 mL aliquots of the culture are centrifuged (Centrifuge 5415, Eppendorf AG, Germany) at 10 000 rpm for 10 min. The supernatant is filtered using a 0.22 µm filter (Spartan 30/0.2 RC, Filter unit, 0.2 µm, 10 463 060, Whatman, Germany). The samples are perfumed on column SUPELCOGEL C610H cation exchange (H^+) with appropriate pre-column, mobile phase (0.00275 mM H_2SO_4 (1.35 mL H_2SO_4 in 5 L UHP water)) with isocratic elution (Flow rate: 0.5 mL/min) , Internal standard: isopropanol 30 [g/L], refractive index detector (positive polarisation). We determine of metabolite concentration by integrating area or height pic in function concentration.

2.6.Optical density

The optical density of 1 mL of sample is read at 600 nm with spectrophotometer (Spectrophotometer Helios Epsilon, Thermospectronic, USA). The absorbance range is 0.1 to 0.8 [-] and dilutions are performed with distilled water.

2.7.Protein determination by Bradford method

1 mL aliquots of the culture were centrifuged (Centrifuge 5415, Eppendorf AG, Germany) 10 000 rpm for 10 min. The Bradford method is performed on supernatant. 20 µL of supernatant and 1 mL Bradford reagent (Bradford Reagent, B6916-500ML, Sigma-Aldrich, USA) are mixed. After 5 min, the absorbance is read at 595 nm with spectrophotometer (Spectrophotometer Helios Epsilon, Thermospectronic, USA). A calibration curve is created with BSA (Albumin Bovine, Initial Fractination By Heat Shock, A-3294, Sigma, Germany) and hSA (Albumin from human serum 97-99%, A9511-1G, Sigma-Aldrich, USA). The concentration range is 0 to 1 [g/L] (Table 2).

Table 2: Protocol for protein determination

Standard	**1**	**2**	**3**	**4**	**5**	**6**
Protein [g/L]	0	0.2	0.4	0.6	0.8	1.0
$V_{Standard}$ [µL]	0	20	20	20	20	20
$V_{Reagent\ Bradford}$ [µL]	1000	1000	1000	1000	1000	1000
Mix and read after 5 min at 595 nm						

2.8.Requirement of histidine

400 mL BSM medium at pH 5 are thermostated at 30 ° C, and 1.74 mL PTM1 and 2 cryotubes of WCB *Pichia pastoris* are added. The suspension is mixed and 100 mL of this suspension are dispended in 4 shakeflasks of 1 L. A histidine solution of 0.4% is added according to **table 3**.

Table 3: Protocol for requirement of histidine

Shakefalsk	Media sterile	1	2	3	4
$V_{Histidine\ 0.4\%}$ [mL]	0	0	0.5	1	10
Incubation 30°C, 200 rpm and 24h					

2.9.Create model for monitoring metabolites

According the literature [21], create model for on-line monitoring glycerol, methanol, ammonium and phosphate by FTIR (ReactIR™ 4000 FTIR, Mettler Toledo, Switzerland). The model has 50 standards (**Annexe 3**). Three methodes are used:

- Methode N°1 : Take spectra water before each 10 standards
- Methode N°2 : Take spectra water before each 5 standards
- Methode N°3 : Take spectra water before each standard

2.10. Off-line determination of protein by FTIR

The solution is prepared in water according **table 4**. Proteins tested are BSA (Albumin Bovine, Initial Fractination By Heat Shock, A-3294, Sigma, Germany), hSA (Albumin from human serum 97-99%, A9511-1G, Sigma-Aldrich, USA), Pepsin (Pepsin from porcine gastric mucosa, 1064 [U/mg protein], P7000-25G, Sigma-Aldrich, USA), Lipase (Lipase from *Candida rugosa*, 4.01 [U/mg], L1754-5G, Sigma-Aldrich, Japan) and α-Amylase (α-Amylase from *Bacillus amyloliquefaciens*, A7595-50ML, Sigma-Aldrich, Denmark). The dilutions are performed in distilled water.

Table 4: Range of concentration for FTIR

Standard	1	2	3	4	5	6	7	8
Protein [g/L]	0.078	0.156	0.313	0.625	1.25	2.5	5	10

Before each series protein take a water spectre.

For this method two FTIR are used for the off-line detection of proteins:

- FTIR type 1 : ReactIR™ 4000 FTIR, Mettler Toledo, Switzerland
- FTIR type 2 : ReactIR iC10® with MCT detector Mettler Toledo, Autochem, K6 conduit 16 mm probe

2.11. Calculation

- The generation time is: t_G [h] = (Ln 2) [-] / μ_{max} [h^{-1}]

- The biomass/substrate yield: $Y_{X/S}$ [g biomass/g glycerol] = ($X_{End\ Batch}$ [g/L]* $V_{End\ Batch}$ [L]) / $S_{Glycerol\ Batch}$[g]
  $Y_{X/S}$ [g biomass/g glycerol] = {($X_{End\ Feed}$ [g/L]* $V_{End\ Feed}$ [L]) - ($X_{End\ Batch}$ [g/L]* $V_{End\ Batch}$ [L])} / $S_{Glycerol\ Feed}$[g]

- The product/substrate yield: $Y_{P/S}$ [g hSA/g methanol] = $m_{hSA\ produced}$ / $m_{Methanol\ injected}$

- The product/biomass yield: $Y_{P/X}$ [g hSA/g biomass] = $Y_{P/S}$ / $Y_{X/S}$

- The specific biomass generation is: r_X [g biomass/L/h] = μ_{max} [h^{-1}] * X [g/L]

- The specific substrate consumption rate is: $-r_S$ [mg methanol/L/h] = r_X / $Y_{X/S}$

- The specific HAS production rate is: (m_P has been neglected in $q_P = \mu_{max} * Y_{P/S} + ms * X$)
  - q_P [mg hSA/g methanol/h] = $\mu_{max} * Y_{P/S}$

- The **specific productivity** is [µg de hSA/10^6 cell/h]
- The **specific volumetric productivity** is [mg de hSA/L/h]
- The **global productivity** is [mg hSA]

3. Results and discussion

In this section we discuss the set-up of the process of the fed-batch cultures of *Pichia pastoris*. We describe the problems encountered and possible solutions. The results will be immediately followed by a discussion.

The following subchapters allow to understand the following sequence of steps: the process set-up with the verification of histidine requirements for the strain, a study of the cloudiness of the medium, the off-line protein detection, the fed-batch cultures and a study on detection of protein by FTIR.

3.1.Process set-up

3.1.1. Requirement of histidine for *Pichia pastoris*

In the literature [27], we can see that the plasmid containing the gene of interest can be kept as it is or integrated into the genome of yeast. In the latter case, integration can be achieved either by homologous recombination of the *his4* or *AOX1* loci. If the integration of *the hSA* gene is in the *his4* locus, the strain lost its ability to synthesize histidine and becomes auxotrophic for this amino acid (his-). But if the integration is performed on the *AOX1* locus, the strain can still synthesize histidine (his +). Thus it is important to control the requirement of histidine for the strain. The results are given in **table 5**.

Table 5 : Requirement of histidine in shakeflask

Shakeflask	Media sterile	1	2	3	4
$V_{Histidine\ 0.4\%}$ [mL]	0	0	0.5	1	10
Incubation 30°C, 200 rpm and 24h					
$OD_{600\ nm}$	1.18	17.9	24.3	17.6	22.5

There has been growth in all shakeflasks. The fluctuations that are observed are probably due to the glass used. Indeed, some shakeflasks have a roughness that others do not have. This may improve mass transfer inside the flasks. Moreover we have seen similar fluctuations in the number of cells injected into the reactor during preculture, even though precultures are all performed under identical conditions. In this particular case of the histidine requirement test, whereas the shakeflask n°1 is devoid of histidine, growth is about the same everywhere. We can thereby conclude that the strain is **not auxotrophic for histidine**.

This information can also allow some hypothesis about the supposed gene for hSA [28]:

- The plasmid is in its circular form and free in the cytoplasm, therefore the histidine gene is active
- The plasmid is integrated by homologous recombination at the AOX1 loci therefore the histidine gene is active
- The strain used does not contain the plasmid vector encoding the hSA

To verify this, it would be wise to make a simple qualitative PCR to check the presence or absence of hSA gene and the copy number.

3.1.2. Media screening

Experimentally and in the literature [27], we see that the medium used is cloudy after the addition of the PTM1 solution. This is problematic because it causes errors in the results. Especially for OD_{600nm} and DCW. It was assumed that this couldiness came from a cloudy state of equilibrium between certain salts of the medium. Thus, it is difficult to assess whether this cloudiness varies significantly during the fermentation. It can be assumed that indeed the cloudiness varies because the cells grow and use the different salts and produce the compounds needed for their development. To better understand this phenomenon, experiments were performed. Initially, we tested the solution of vitamins (PTM1) then in the media. Then, we used EDTA to counteract the disorder.

Experience n°1:

A 4.35 mL of PTM1 are added to 1 L of H2O. The solution is clear and the pH = 2.3. The pH is adjusted to 5 with NaOH 4M. The solution becomes yellow-orange with a medium turbidity. The same phenomenon occurs when using NH_4OH 8M as base for pH correction. Figure 8 shows the aspect of the solutions after adjusting the pH.

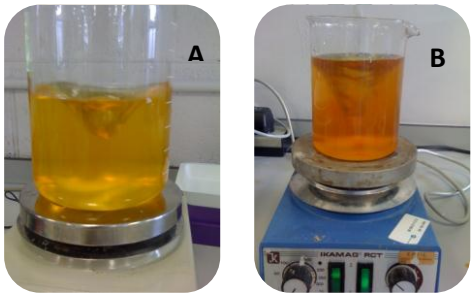


Figure 8 : A) Solution after NaOH used. B) Solution after NH_4OH used

The disorder appears only when the pH is modified by base addition. We can therefore assume that the ionization state of some compounds, including iron for instance, precipitate as a function of the pH.

EDTA, known for its characteristics as an ion chelator, is used to address this issue. The first steps of the experiment allow knowing the critical concentration of EDTA where the cloudiness makes it disappearance. All experiments are carried out in 1 L of BSM adjusted to pH=5 with NaOH 4M. 4.35 mL of PTM1 are added to this solution (Figure 9).

Figure 9: 1 L BSM pH=5 and 4.35 mL of PTM1

Experience n°2:

- 0.04 g of EDTA are added to 50 ml of BSM. The solution is still cloudy.
- A few millilitres of concentrated H_2SO_4 are added to this new solution this solution becomes clear and limpid.

The cloudiness of the solution is dependant of the pH.

Experience n°3:

- A few millilitres of concentrated H_2SO_4 are added to 50 ml of BSM. The mixture becomes clear and the measured pH is equal to 1.5.
- A few millilitres of 4M NaOH are then added and the solution becomes cloudy again and the measured pH is 4.9.

This test confirms that the pH is crucial to the appearance of the cloudiness of the solutino. It is clear that the steady state is disrupted provoking the precipitation of salts.

Experience n°4:

80 g of EDTA are added to 80 ml of BSM. The solution is still cloudy, but after 30 min of stirring, the solution becomes clear and the pH = 3.8.

Experience n°5:

A few millilitres of concentrated H_2SO_4 are added to 75 ml of BSM. The pH = 1.6 and the solution is clear. A few drops of 4M NaOH are added and the pH = 2.7 and a slight haze appears.

Experience n°6:

Different concentrations of EDTA are tested and the results are shown in **table 6**. For information, the pH is only measured, not adjusted when adding EDTA.

Table 6 : Visualisation of the turbidity after the action of EDTA

	1	2	3	4	5	6
V_{BSM} [mL]	75					
EDTA [g]	0	0.005	0.01	0.02	0.03	0.04
Turbidity (Figure 10)	+++	+++	+++	+++	+++	+++
Overnight under mix						
Turbidity (Figure 11)	+++	+++	+++	++	+	-
pH [-]	4.77	4.66	4.55	4.33	4.15	3.87

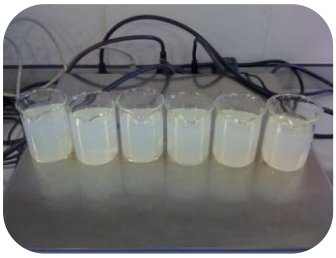
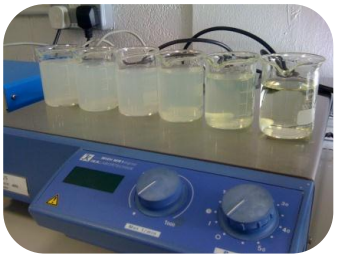


Figure 10 : Turbidity at t_0



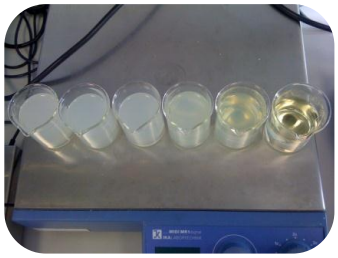


Figure 11 : Turbidity after overnight under mix

It is found that 0.04 g EDTA are sufficient to remove the cloudiness in 75 mL (EDTA 0.53g / L BSM) completely. We also note that the more EDTA is added, the more the pH decreases. The question to ask is whether the pH is responsible for the disappearance of cloudiness or if it is only due to the chelating action of the EDTA solution, or if it is a combined effect.

Experience n°7:

To confirm the previous result, 370 mL of BSM are mixed with 0.1998 g of EDTA (0.54 g EDTA / L BSM). **Figure 12** shows the evolution of the cloudy as time.

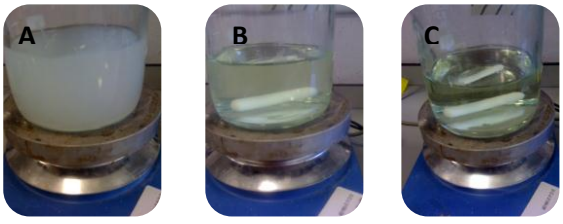


Figure 12: A) At t=0 h. B) at t=1h06. C) At t=1h45.

We note the same outcome; the cloudiness of the solution disappears after 53 min of stirring. Thus, EDTA appears to be a good agent against the cloudiness issue. It is therefore decided that 0.54 [g/L] of EDTA will be to use in culture in a first trial.

3.1.3. Culture in RC1 using EDTA

A *Pichia pastoris* strain was cultivated in a calorimetric reactor (modified RC1, Mettler Toledo, Greifensee, Switzerland). The medium was 1.3 L BSM and contained 0.6933 g EDTA (0.53 [g EDTA/L BSM]). The medium is clear and translucent. 100 mL of YPGlycerol (6 [g/L] Yeast extract, 5 [g/L] Peptone, 20 [g/L] Glycerol) were inoculated with 1.8 mL WCB *Pichia pastoris GS115*. The number of viable cells inoculated was **$1.7*10^8$ cells**. The culture conditions remained unchanged to compare section material and methods.

After 5 hours of culture, there was still **no growth**. We supplemented the medium with 5 mL of PTM1. But three hours later, still no growth. It was assumed that EDTA used at this concentration could inhibit the growth of yeast cells. Indeed, the main role of EDTA is its ability to form a complex with cationic ions. We assumed that the ion most likely to be chelated with EDTA would be the iron ion. By observing the composition of the solution PTM1, there is a very large amount of iron. Given the content, we assumed that the cloudiness forms due to the addition of this high content of iron in the medium that interacts with other salts. However, it is clear that EDTA may well chelate other ions such as Mg^{2+} ion necessary for the proper functioning of enzymes for metabolism and cell growth.

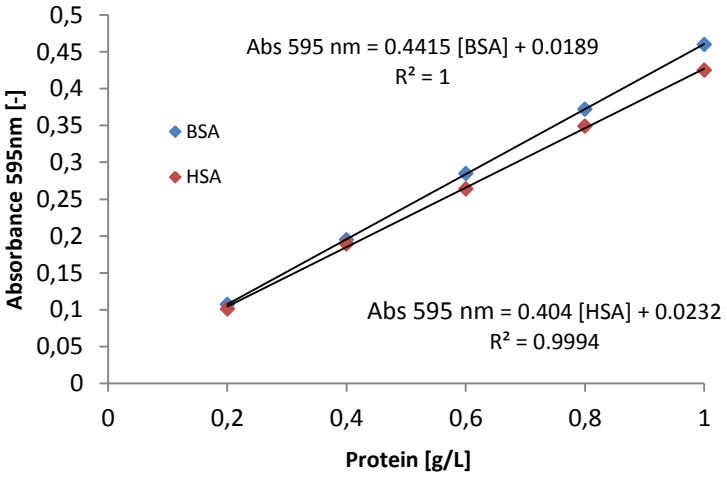
In this sense, two experiments should be considered to resolve this problem. Initially, other ion chelators less toxic and whose molecular composition does not significantly influence growth should be tested. On the other hand the concentration of iron in the solution PTM1 could be decreased ensuring however that there is no iron-limitation. So given the toxicity of EDTA, it will not be used in future fermentations.

3.1.4. Protein detection (Off-line)

In this section we have studied two methods to quantify protein production.

Figure 13 shows the calibration of protein by the Bradford method using a conventional spectrophotometer. There is a difference in the responses, the literature exposes the fact that BSA provides answers in terms of protein concentrations that are overestimating the samples because of the difference in amino acid composition. Thus, in order to represent as accurately as possible the reality, we take the hSA as a reference for the development of a calibration curve for the quantification of proteins.

Figure 13 : Calibration BSA and HSA using conventional spectrophotometer



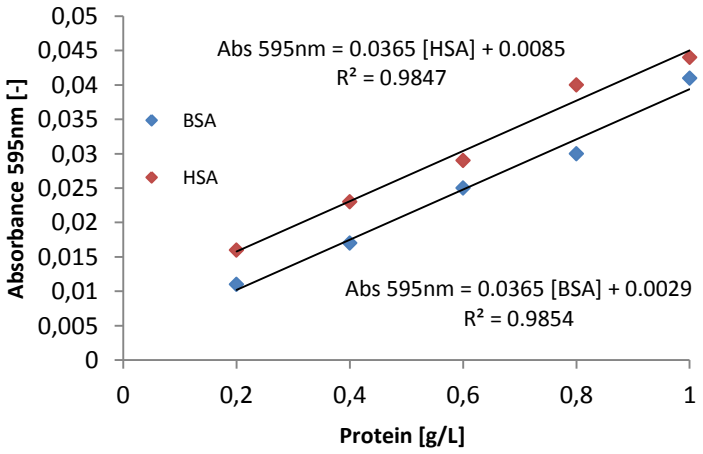


Figure 14 shows the calibration of protein using the Bradford method but with a NanoDrop® for the detection. There is a relatively good correlation, but weaker than with the conventional Bradford method involving a standard spectrophotometer. A more noticeable difference in the responses of the two proteins can be observed in this case.

Figure 14 : Calibration BSA and HSA using NanoDrop®

3.1.5. Fed-Batch

In sub-sections that follow we will discuss the fed-batch that have been made, we will highlight the problems encountered and possible solutions are listed.

3.1.5.1. PpasFB01

The preculture was performed in 100 ml of YPG at pH = 5 with one WCB cryotube at 30°C, 200 rpm for 24 h. For the first fermentation, we used the Egli medium contain **20 [g/L] glycerol** and a vitamin and trace element solution already used beforehand for another strain of *Pichia pastoris* producing avidin in the laboratory (**Annexe 4**).

No growth was observed after 15 hours of culture at 30°C and pH = 5. The culture medium and/or vitamin solution used is probably not suitable for the growth of this strain. Indeed, growth in medium YPG is very good. So the viability of the strain is not be blamed. However we see that the vitamin solution contains EDTA, this product seems to inhibit the growth of this strain. This result comes from the culture RC1 visible in section **3.1.3**. So according to this result, it was decided to change the media and vitamin solution. To do this we followed the guideline on the fermentation conditions of *Pichia* strain by Invitrogen **[27]**.

3.1.5.2. PpasFB02

We found the BSM medium used by Invitrogen is devoid of nitrogen source. Indeed in there culture conditions, the nitrogen source comes from the base used to correct the pH. We supplemented with NH_4Cl. The medium contain per litre 13.35 mL H_3PO_4 85%, 0.59 g $CaSO_4$ $2H_2O$**,** 9.10 g K_2SO_4, 7.45 g $MgSO_4$ $7H_2O$, 2.06 g KOH, **10 g Glycerol**, 9 g NH_4Cl. The glycerol feed contains per liter **300 g glycerol** and 44 g NH_4Cl.

At the end of the preculture, we got a concentration of $2.7*10^6$ [cells/mL] and a viability of 82%. The reactor was inoculated with Bioeng **$1.8*10^9$ viable cells** in 2 L of BSM medium.

After 10 h of batch, there is still no growth, but we note that the air outlet filter was blocked. The reactor pressure was at its maximum and the mass flow controller did not provide more air because of this pressure. After removing the filter, pressure and air flow went back to normal conditions. Growth could begin right after this incident. The **figure 15** shows the profile O_2% and CO_2% during fermentation. We can easily find the indications of the blocked filter in the data acquired through the gas analyser. However we see that the profile of oxygen is increased, this may be due to a failure in the gas analyser calibration and/or because of that pressure. Indeed, the gas analysis is composed of a electrolytes to detect the oxygen. Values are probably biased, but the profile can still be studied for a first estimate of the characteristics of the strain. Thus, we can say at first that the culture environment is good for growth. On the other hand, the glycerol feed phase allows greater production of CO_2, suggesting that the process is adapted to the strain.

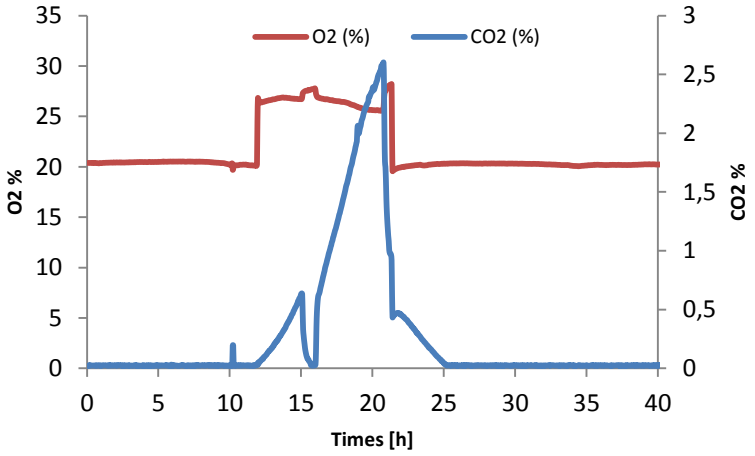


Figure 15: Profile O_2% and CO_2% gas outlet during fermentation

The PLS model "Monitoring PP" used to transcribe the FTIR spectrum determines properly 10 [g/L] of glycerol at the beginning of the batch (figure 16). It is found that the batch phase ends at 15 h, but the glycerol concentration is not 0. It is possible the model is not accurate for lower concentrations of glycerol. **Biomass is 6.3 [g/L], $Y_{x/s}$ = 0.64 [g/g], μ_{max} = 0.26 [h^{-1}], t_d = 2.67 [h], r_x = 1.64 [g/L/h], $-r_s$ = -2.56 [g/L/h] and q_s = 0.41 [g/g/h]**. The yield is similar to literature [29] but the specific growth rate is better in this process because glycerol concentration is not potentially inhibiting [27, 29]. Glycerol feed is started at 15.21 [h] and finished at 20 [h]. The model determines the increase of glycerol in the reactor. When the feed is stopped we have a decrease glycerol concentration in the medium. We subsequently found that during the induction phase with methanol, the glycerol concentration increases. This is due to the method used. We used the bottle of feed glycerol to induce with methanol where rests of glycerol were still present in the lines.

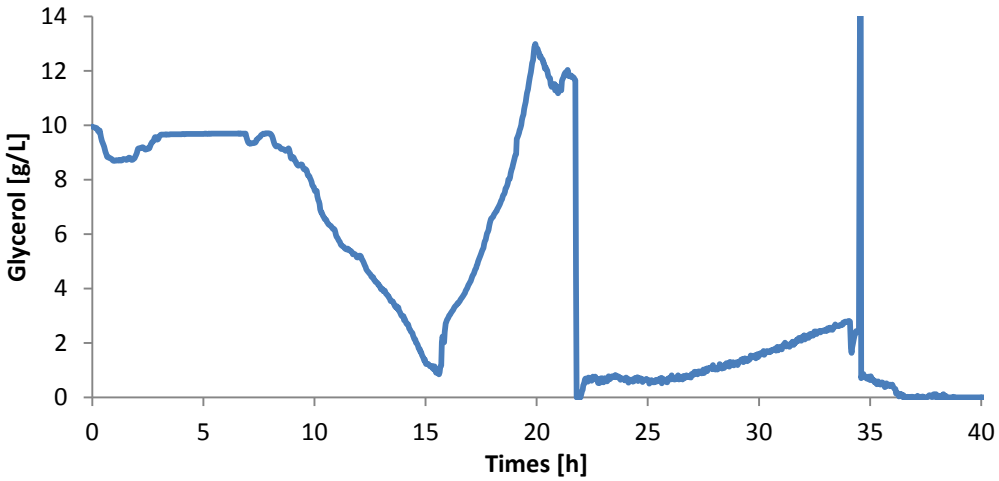


Figure 16: Evolution glycerol concentration by FTIR

A significant fact was also revealed during this fermentation. To convert the units given by the capacitance reading C [pF/cm] to Biomass X [g/L], a **conversion factor of 4.82** was introduced in Labview. This factor comes from a fermentation conducted by Michal Dabros and Moira Schuler (Michal Dabros, Labbook D66174). The figure 17 shows the evolution of biomass given via the capacitance during fermentation. One can see 3 parts: the lag phase which lasts until about 8-10 h due to the pressure, batch phase and feed glycerol phase. In this latter phase, it is found that the maximum value of **biomass is only 33 [g/L], $Y_{x/s}$ = 0.48 [g/g], μ_{max} = 0.22 [h^{-1}], t_d = 3.15 [h], r_x = 5.87 [g/L/h], $-r_s$ = -12.23 [g/L/h] and q_s = 0.45 [g/g/h]**. To increase this value, the 500 mL of glycerol feed will be more concentrated from 300 to 500 [g/L] for the next fermentations.

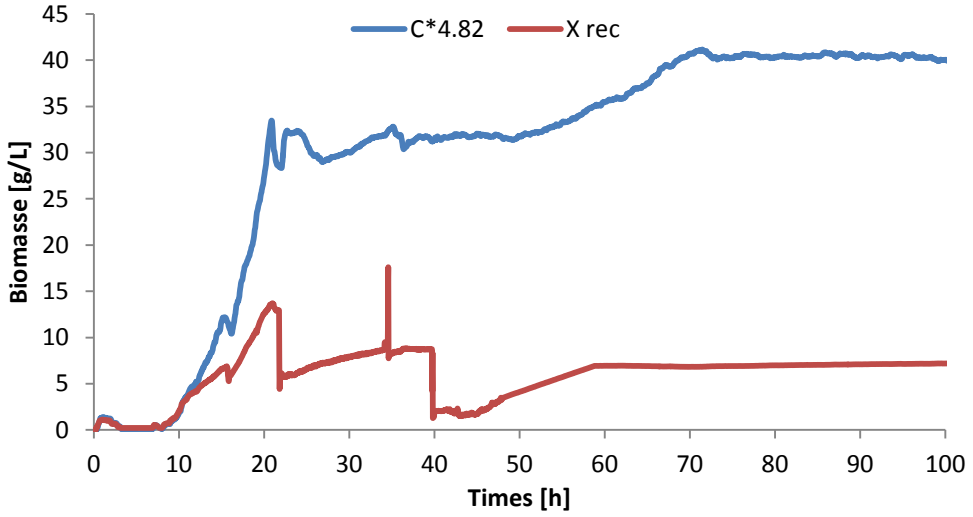


Figure 17: Compare between biomass reconciled and biomass by capacitance

We can see different values between biomass. There are similar values until 11 h. We suppose the capacitance is closer to the true values. Indeed, data reconciliation received several data given from different tools. It is likely that if one tool sends bad data that this disrupts smooth functioning of elemental and mass balancing. We have seen the FTIR is not accurate at low concentrations and that the gas analyser is not calibrated correctly. The recognition is affected and seems to be incapable to reconcile the values thereafter.

After 3 days of induction with methanol at 0.025 [g/min], no detection of proteins has been revealed by the Bradford method. But this strain grows on methanol and the final **biomass** is **40 [g/L]**. The **figure 18** shows the supernatant of sample collected at the end of the batch, of feed glycerol and methanol feed. We can see that the tube n°3 has a yellowish colour which may suggest the production of the protein at a very low concentration. This can be explained by the induction method; in fact we have introduced the methanol into the bottle used to feed the glycerol. It is likely that glycerol is still able to play a role in repressing the production of hSA.

Figure 18: Tube n°1 is supernatant at batch end, tube n°2 at feed glycerol end and tube n°3 at fermentation end

So for the next fermentation:

- Check the pressure in the reactor
- Use a glycerol feed of 500 [g/L]
- Induce methanol with a new bottle

3.1.5.3. PpasFB03

We have doubled the volume of preculture, the reactor was inoculated with **$2.1*10^9$** of viable cells. The culture conditions are always the same (30°C, 800 rpm and pH = 5). After 10 hours of culture, we see the **same problem** of pressure in the reactor and therefore **no growth**. We have established that gas analysis is certainly responsible for the increase in pressure. Here is the detailed reasoning about the problem in question:

- **Mass flow controller**: it cannot be the cause because it works perfectly during the leak test and the more it feeds back the air when the air out is disconnected.
- **Tube-filter air inlet**: This part seems to be problematic because after a few hours of fermentation the problem appears
- **Condenser**: After dismantling and cleaning no aggregates that could create a pressure in reactor can be found.
- **Tube-filter air outlet**: no pressure is detected off-line, air passes very well through the bottle and filter
- **Gas analyser**: it passes the calibration successfully but we note that the profile of the voltage fluctuates compared to last calibrations. In addition, when it is disconnected, the pressure in the reactor back to normal. It is very likely that a safety valve snaps into the unit and cut the entrance of air into the gas analyser. This break seems to occur after several hours and not after inoculation.

We have disconnected the air outlet gas analyser and the cell growth. But the biomass monitor shows a negative value until 20 [h]. We obtain a **biomass** at end glycerol feed of **50 [g/L]**. A marked improvement in the final fermentation by the use **feed glycerol** to **500 [g/L] can be noted**. However, after induction with methanol, we do not get high enough titre of hSA to be detected by the Bradford method. The strain used here comes from the laboratory of EPFL (Lausanne, Switzerland). No identification could be made beforehand to ensure the family, genus and species of yeast. Furthermore, the number of copies in the genome of the gene expressing the protein hSA can be critically It will be beneficial to establish a clear identification of the strain and to determine the presence and/or gene copy number **[26, 29]**. Because the laboratory does not have to date equipment and resources necessary for the establishment of these manipulations, we will work for the next fermentation with a new strain of *Pichia pastoris* from the HES-SO (Sion, Switzerland): *Pichia pastoris GS115*/Mut^S/his-/sechSA.

3.1.5.4. PpasFB04

In a first step, we checked the histidine requirements for this new strain. Then we performed a fermentation with the improvements previously suggested. The **table 7** shows the result for auxotrophy.

Table 7: Requirement of histidine

Shakeflask	Media sterile	1	2	3	4
$V_{Histidine\ 0.4\%}$ [mL]	0	0	0.5	1	10
Incubation 30°C, 200 rpm and 68 h					
$OD_{600\ nm}$	1.18	18.7	19.3	19.1	18.2

We observe in shakeflask 1, 2, 3 and 4 growths as expected. $OD_{600\ nm}$ values are similar and we can conclude that the strain is **not auxotrophic for histidine**. The same hypothesis about the hSA gene can be written, as already stated in section **3.1.1 [28].**

The 2 L medium before inoculation is the usual BSM medium and contains 8.7 mL PTM1 solution and 1 mL of PPG. The BSM medium contains **10 [g/L] glycerol** and **glycerol feed 500 [g/L]**.

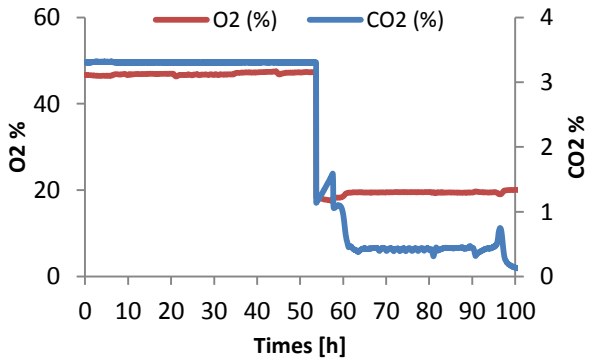


Figure 19 shows the evolution of O_2% and CO_2% at the reactor outlet. It is found that the profiles are incorrect. It seems that gas analysis is faulty. It is possible that the effect of pressure on the device has compromised the reliability.

Figure 19: Profile O_2% and CO_2% gas outlet during fermentation

Figure 20 shows that the batch phase lasts only 6.6 hours. It can be seen in figure the value of **biomass** is **2.4 [g DCW/L]**. The yield is **$Y_{x/s}$ = 0.24 [g/g]**. The medium is suitable for the growth of the strain.

The feed started only at 9 h with a rate of 0.3 [g/min] inserted manually in LabVIEW (**Figure 21**). This problem is caused by LabVIEW. Indeed, the program is built in such a way that in principle there is an automated calculation of the factor F0 to start the exponential feed (**Equation 3**). However, it appears that F0 is not calculated.

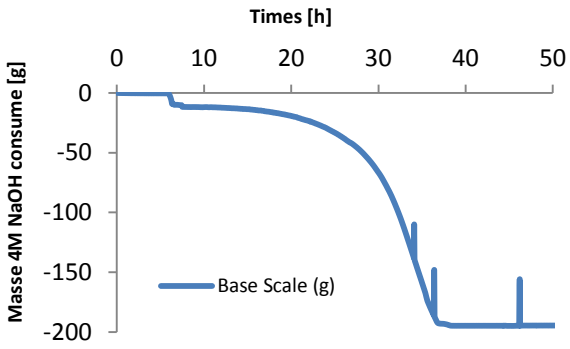
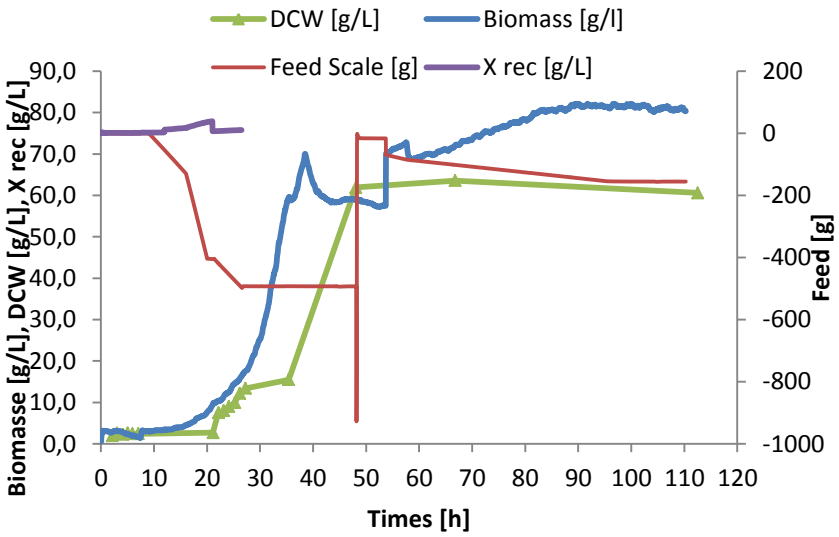


Figure 20: Consummation of NaOH 4M during fermentation

After 26.59 h of culture, the feed is stopped. The pO_2% was below 20%, we increased agitation to increase the pO_2% between 70-80%. After 35.39 pm pO_2% is 0%. This suggests that the feed rate is too fast. A high accumulation of glycerol in the reactor occurs. Despite an increase from 1300 to 1500 rpm, pO2% is always 0%. We harvest 350 ml of culture because rich foam is created with the agitation that can block the air filter.



At 48.04 h, pO_2% shows 100%. This indicates shows that glycerol accumulates in reactor. The **biomass** is **62 [g DCW/L]**.and **$Y_{x/s}$ = 0.41 [g/g]**, **μ_{max} = 0.12 [h^{-1}]**, **t_d = 5.78 [h]**, **r_x = 7.15 [g/L/h]**, **$-r_s$ = -17.44 [g/L/h]** and **q_s = 0.29 [g/g/h].**

Figure 21: Monitoring Capacitance, DCW and Feed scale during fermentation

The model "Monitoring PP" detects 10 [g/L] glycerol after 12 [h] but the concentration is 0. This is impossible because in figure 20 the batch is finished at 6 h, it seems that it is a problem of data acquisition by MatLab or rather a problem of accuracy in the created model.

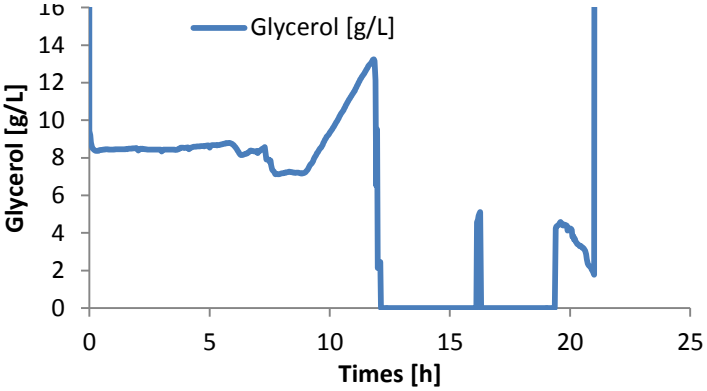


Figure 22: Evolution of glycerol concentration by FTIR

The **table 8** shows the protein concentration during fermentation. The methanol feed began at **48.28 h** with a rate of 0.023 [g/min] (= 0.9 mL/h/L fermentation initial). It is found that the **concentration of hSA** of **0.27 [g/L]** is very low after 112.5 h and the **specific volumetric productivity** is **2.4 [mg hSA/L/h], the global productivity is 567 [mg hSA], $Y_{P/S}$ = 0.0064 [g hSA/g methanol], $Y_{P/X}$ = 0.0045 [g hSA/g biomass], $Y_{X/S}$ = 1.42 [g biomass/g methanol], q_P = 0.0704 [mg/g/h].** Moreover we cannot say that this low rate is only due to the expression of hSA. Indeed, it is very likely that methanol is able to cause cellular damage. Proteins have been released and caused an increase of absorbance in the Bradford method. Thus it is possible that the concentration of hSA is overestimated.

Table 8: Production of protein after induction

Times [h]	21.11	24.09	27.24	48.04	66.78	112.49
OD_{595nm} [-]	0.007	0.009	0.004	0.023	0.058	0.132
BSA [g/L]	0	0	0	0.01	0.09	0.26
hSA [g/L]	0	0	0	0	0.09	0.27

It is possible that the accumulation of glycerol is stressing the cells. The methanol feed is initiated only 0.3 h after end consumption of glycerol accumulated. The glycerol feed seems too fast for the biomass at the end batch. Thus we must increase the value of the biomass at the end of the batch. The **medium BSM** will **contain glycerol 40 [g/L]**.

3.1.5.5. PpasFB05

The figure 23 shows evolution of the gas out and the figure 24 shows the concentration of glycerol and biomass. It was found that the batch phase lasts 22 h. Biomass amounted to **31.6 [g DCW/L]**, **$Y_{x/s}$ = 0.8 [g/g]**, **μ_{max} = 0.15 [h^{-1}]**, **t_d = 4.62 [h]**, **r_x = 4.74 [g/L/h]**, **$-r_s$ = 5.93 [g/L/h] and q_s = 0.19 [g/g/h]**. It is found that the phase lag is long enough. This can be clearly defined with the profile of the off-gas analysis because we do not take into account the detection limit of gas analysis. The cells may very well start to grow without the produced amount of CO_2% at high enough concentrations to be detected.

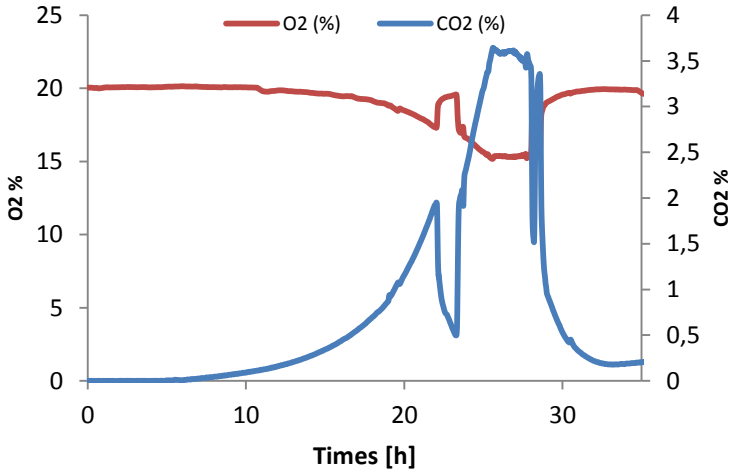
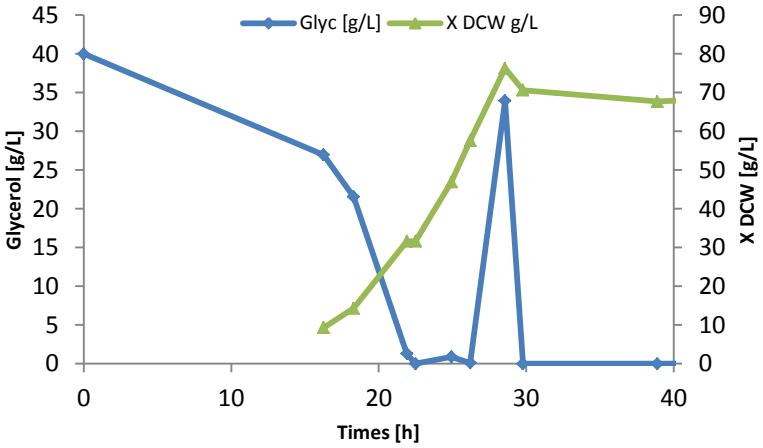


Figure 23: Profile O2% and CO2% gas outlet during fermentation



To reduce the lag phase, we will test for the next fermentation to carry out the preculture in BSM medium. Maybe the adaptation to the culture conditions stresses the cells. If the cells are adapted to the culture medium early enough, there might be a faster start of the batch.

Figure 24: Evolution of glycerol and DCW

The feed has been launched at 23.2 h with **μ_{sp} = 0.3 [h^{-1}]**. F0 factor was calculated incorrectly as follows:

Knowing that **$Y_{x/s}$ = 0.5 [g/g]** and that it has **80 g of glycerol**, we should get **40 g of biomass**. We wish μ_{sp} = 0.3 [h^{-1}] which gives a doubling time of **139 min**. So it would introduce 80 g of glycerol in 139 min, which gives a rate of **0.57 [g/min]**. The error is here that the inserted value for F0 was **4.4 [g/min]**. The feedback control was not initiated because of the capacitance values fluctuated widely. At 28.1 [h] of fermentation, we see that the feed was stopped. We restart with an **F0** of **18.5 [g/min]**. We notice an accumulation of glycerol in 3.5 hours (**figure 25**). The glycerol feed was stopped at 28.6 h. During the feed phase a strong appearance of foam can be noticed due to the increase of agitation necessary to provide oxygen to the cells. It would be wise to add a greater amount of PPG2000 in the initial medium or in to the glycerol feed.

At 30.1 h, the **methanol feed** was started with a rate of 0.072 [g/min] (= 2.84 mL/h/L initially). Visually it is observed that the needle used for feed was large and large drops accumulate. This could be problematic because when the drop falls into the medium, the local methanol concentration is high and could be toxic to the cells. For the next fermentation, we used a finer needle. It can be seen from the **figure 26** that the production of CO_2 increases with time but remains fairly low. It is still assumed at that

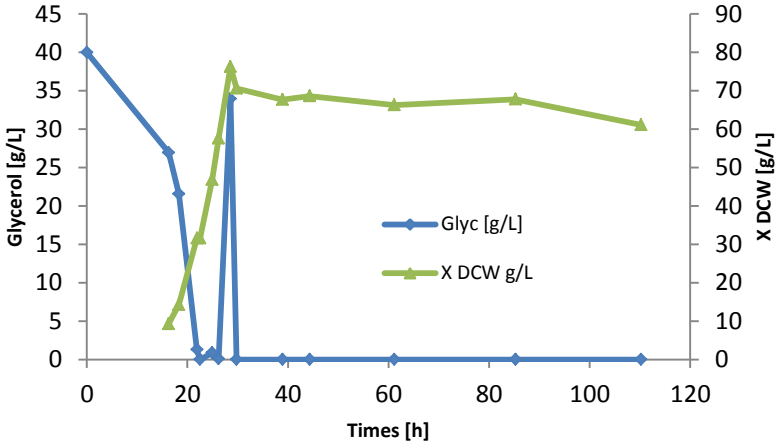


Figure 25: Evolution of Substrate and DCW during the fermentation

stage that the genetic profile of the strain that is Mut^{S}. Furthermore we can see from the **figure 25** that the biomass remained approximately stable. There is still a slight decrease towards the end of the process. At 61.1 h, the temperature is **36°C** in reactor. This is due to a **failure** of the cooling system. We also found that the air filter blocked quite regularly after induction with methanol. It could be argued that methanol evaporates too quickly, despite the presence of the condenser. This phenomenon still occurs after induction and no similar problem is found during the batch phase and feed glycerol. The filter was changed at 61, 63, 85 and 110 hours of culture. It will be interesting to analyse the filter properties to understand this phenomenon.

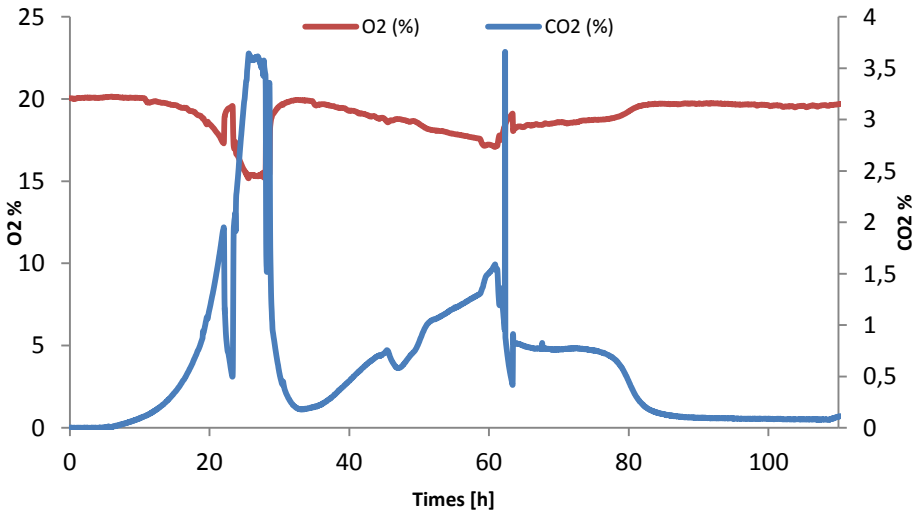


Figure 26 : Profile O2% and CO2% gas outlet during fermentation

The **table 9** shows changes in protein production. It is found that the highest concentration is **0.11 [g/L]**. Fermentation in the previous title was **0.26 [g/L]**. The **productivity** is **3.84 [mg hSA/h]** (using 0.11 [g/L]). The **specific volumetric productivity** is **1.3 [mg hSA/L/h], the global productivity is 232 [mg hSA], $Y_{P/S}$ = 0.0017 [g hSA/g methanol], $Y_{P/X}$ = 0.0016 [g hSA/g biomass], $Y_{X/S}$ = -0.036 [g biomass/g methanol], q_P = [mg/g/h].** This difference may be explained by the rise in temperature to 36 ° C recorded at 61.1 [h]. Since the expression is very temperature sensitive and should not exceed 32°C during induction. Maybe proteases are moreover produced and more active at this temperature **[27, 30]**.

Table 9: Evolution of protein

Times [h]	29.77	38.88	44.32	61.16	85.25	110.227
OD_{595nm} [-]	0.002	0.033	0.042	0.048	0.067	0.048
BSA [g/L]	0	0.03	0.05	0.07	0.11	0.07
hSA [g/L]	0	0.02	0.05	0.06	0.11	0.06

3.1.5.6. PpasFB06

Figure 27 shows the evolution of the process until 30 h. We find that the lag phase is smaller than before. So the use of BSM medium for preculture seemed beneficial to the cell adaptation to the new environment. The batch phase ends to 20.6 h, **biomass** is **29.3 [g DCW/L]**, $Y_{x/s}$ **= 0.75 [g/g]**, μ_{max} **= 0.187 [h^{-1}]**, t_d **= 3.71 [h]**, r_x **= 5.48 [g/L/h]**, $-r_s$ **= -7.31 [g/L/h] and** q_s **= 0.25 [g/g/h]**. There are good correlations with data from the previous process.

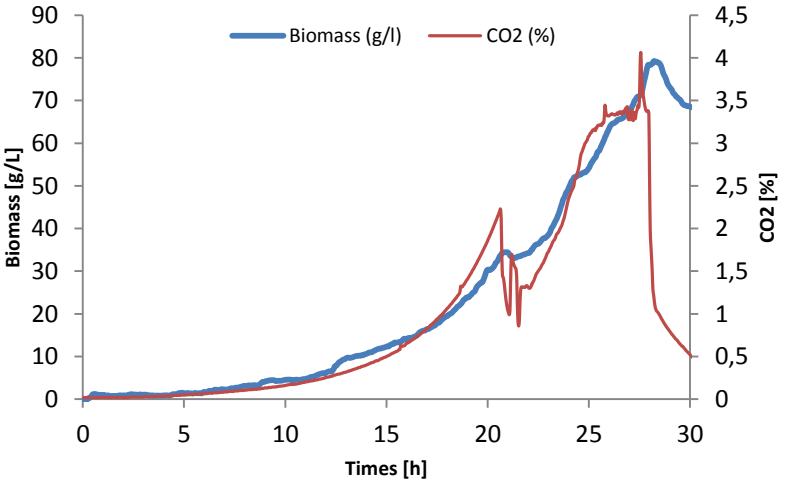


Figure 27 : Evolution of biomass and CO2% during batch and feed glycerol

Feed glycerol was started at 21.50 h with F0 = 0.3296 [g/min], μ_{sp} = 0.3 [h^{-1}]. The feed was stopped at 27.93 h because pO_2 = 30 %, T=32°C and there was a big quantity foam apparent (dangerous if the condenser is blocked). The foaming may be due to a very high process fluid viscosity and the metabolism of the cells releases a lot of heat. However, when we stop the feed, the quantity of CO_2 produced decreases. So we conclude the feed exponential (**equation 3**) appears to be functional. At this time the **biomass** is **76.6 [g/L]**, μ_{max} **= 0.137 [h^{-1}]**, $Y_{x/s}$**= 0.82 [g/g]**, t_d **= 5.06 [h]**, r_x **= 6.48 [g/L/h]**, $-r_s$ **= -7.90 [g/L/h] and** q_s **= 0.17 [g/g/h].**

Figure 28 shows the glycerol consumption and reconciled biomass values. The model "Monitoring PP" used is very accurate because it predicts the glycerol concentration at 40 [g/L] at the beginning of the culture. But we can see when batch is finished, there is glycerol left according to the values obtained through the model. That is impossible because the CO_2 profile (**figure 27**) shows that the batch finished at 20.6 h. It's sure that there are communication problems between MatLab, Labview and ReactIR or rather interpretation problems due to different errors from the different devices leading to inaccurate data reconciliation. The statistical test of the balancing needs to be checked in detail to address this issue. 40 [g/L] of glycerol are consumed in 20.6 [h], in principle 20 [g/L] are consumed in 15 [h]. But at this time MatLab predict just 28 [g/L] in the reactor. We suppose that the longer the FTIR is in use, the lower is the accuracy of the acquired data. Maybe the light must be changed. Indeed, after discussion with the person from Mettler responsible for the FTIR device, we discovered that during the last FTIR check, it has been highlighted that the light should be changed because it has reached the end of its life.

With this problem the data reconciliation is inexact. We can see that the reconciled biomass values are not true when comparing the data of **figure 27**. There is lot of disturbance.

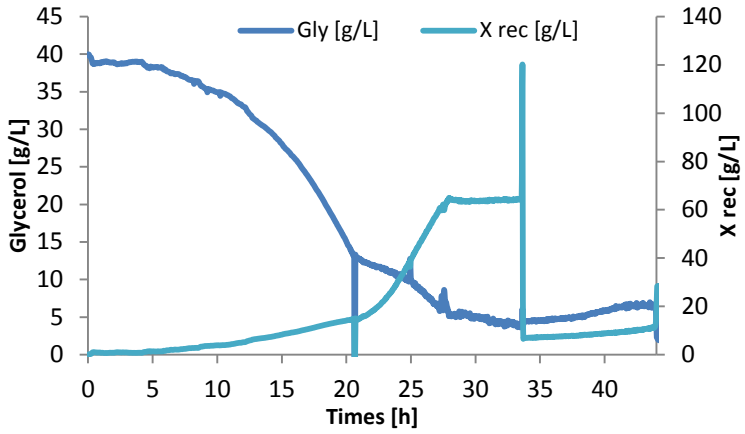


Figure 28 : Profile glycerol by FTIR/MatLab and biomass reconciled

Feed methanol was started at 33.82 [h] with constant feed F0 = 0.0234 [g/min] (= 0.9 [mL/h/L]). At 37.92 [h] F0 is increased to 0.06 [g/min] (= 2.4 mL/h/L]). We note in figure 29 the production CO_2 starts between 35 and 40 [h]. The adaptation to the new carbon source is fast. Then a large amount of CO_2 is produced between 40 and 60 [h] and the value of the biomass is stable. After 60 [h], the production is stable and constant, but there is an increase in biomass. The profiles of CO_2 and biomass are in contradiction. However we can explain this by observing the composition of the methanol feed. It contains the PTM1 solution. It is likely that this addition induces the formation of precipitates. If the precipitates have a polarising affinity, it is possible that the biomass monitor overestimates the value of the biomass.

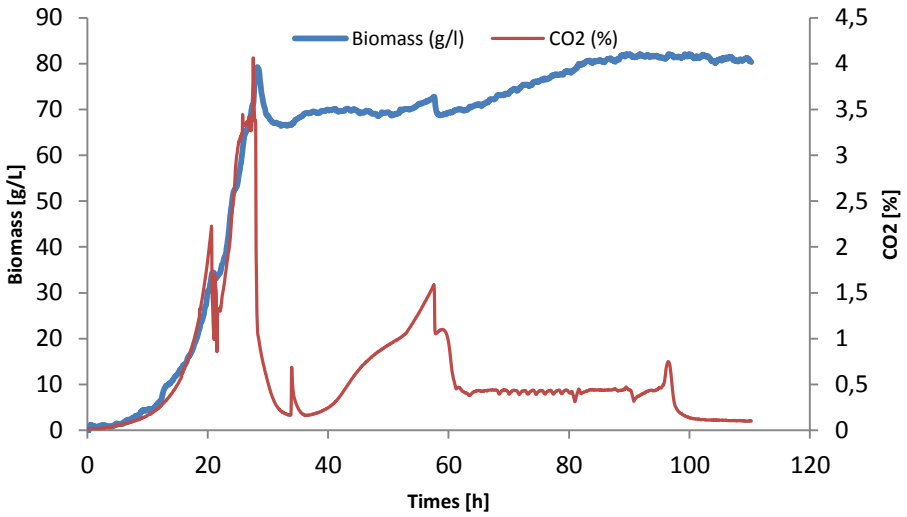


Figure 29 : Evolution of biomass and CO2% during the process

After 115 [h], we noted that the production of hSA is almost equal to 0 [g/L] (Table 10). Two possibilities can explain this phenomenon. The first possibility is that the methanol feed is launched too soon after stopping the glycereol feed. Hence the crucial importance in this kind of process is to follow on-line the concentration of glycerol since it represses the induction of the gene expression. The second possibility is that the addition of methanol was too fast for the cells, the concentration of methanol in the mixture beginning to be toxic. The cells are overstressed and cannot produce the hSA.

Table 10 : Protein concentration at end process

Times [h]	20.87	155.03
OD_{595nm} [-]	0	0.027
BSA [g/L]	0	0.02
hSA [g/L]	0	0.01

For next fermentation:

- Change colony plate *Pichia pastoris*
- Follow scrupulously the Invitrogen guideline for methanol feeding

3.1.5.7. PpasFB07

In this culture we changed WCB. Indeed, given that the production of hSA was low, we selected a pool of colonies from the plate containing *Pichia pastoris* in order to visualize the presence of a colony capable of producing hSA. In addition we have filtered the medium and the glycerol feed after to add solution PTM1.

We can see from **figure 30** and **31**, the time required for the batch phase is 19 [h]. **Biomass is 31.8 [g/L], μ_{max} = 0.18 [h^{-1}], $Y_{x/s}$= 0.78 [g/g], t_d = 3.85 [h], r_x = 5.72 [g/L/h], $-r_s$ = -7.34 [g/L/h] and q_s = 0.23 [g/g/h].** These results are comparable to previous fermentations. It was found that biomass monitor showed negative values at the start of the process. This is normal because inoculation could not be made until one hour after the start of data acquisition. Since the medium is filtered it allowed the study of the optical density and the dry cell weight with less fluctuation. In the early batch phase (5-10 [h]), only the Ln(OD) and Ln(DCW) appear to follow the growth profile while Ln(Biomass) is different. After 10 [h], the three profiles have the same trend. We can also see at the end of batch that the CO_2 profile fluctuates and stays. We assume that some nutrient is likely to have been retained during the filtration in the cake. At 19.7 [h] 9 g of NH_4Cl are therefore injected in reactor. Small peaks appear in the profile but do not influence the values of biomass.

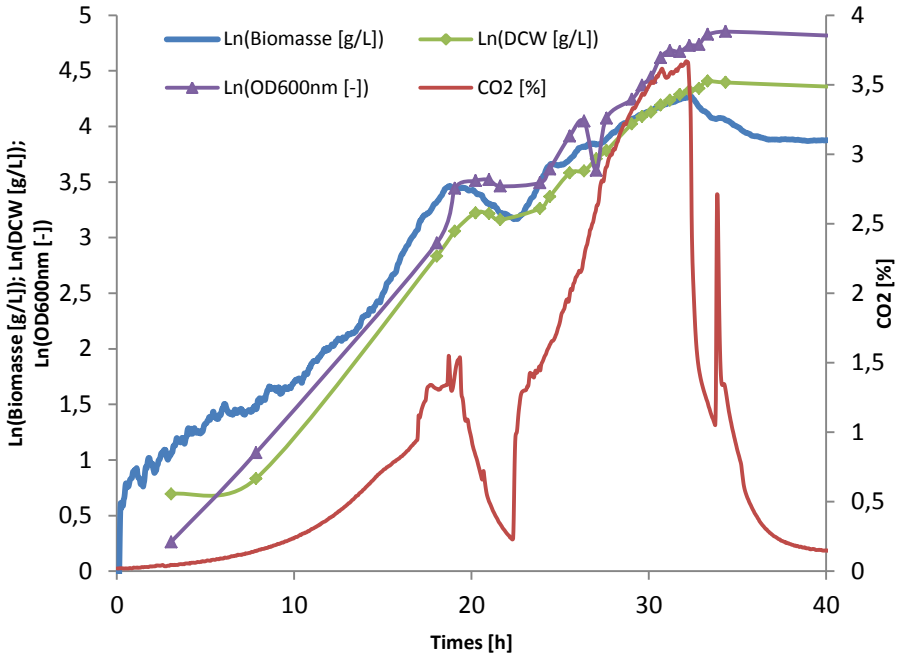


Figure 30: Evolution of biomass after 40 [h]

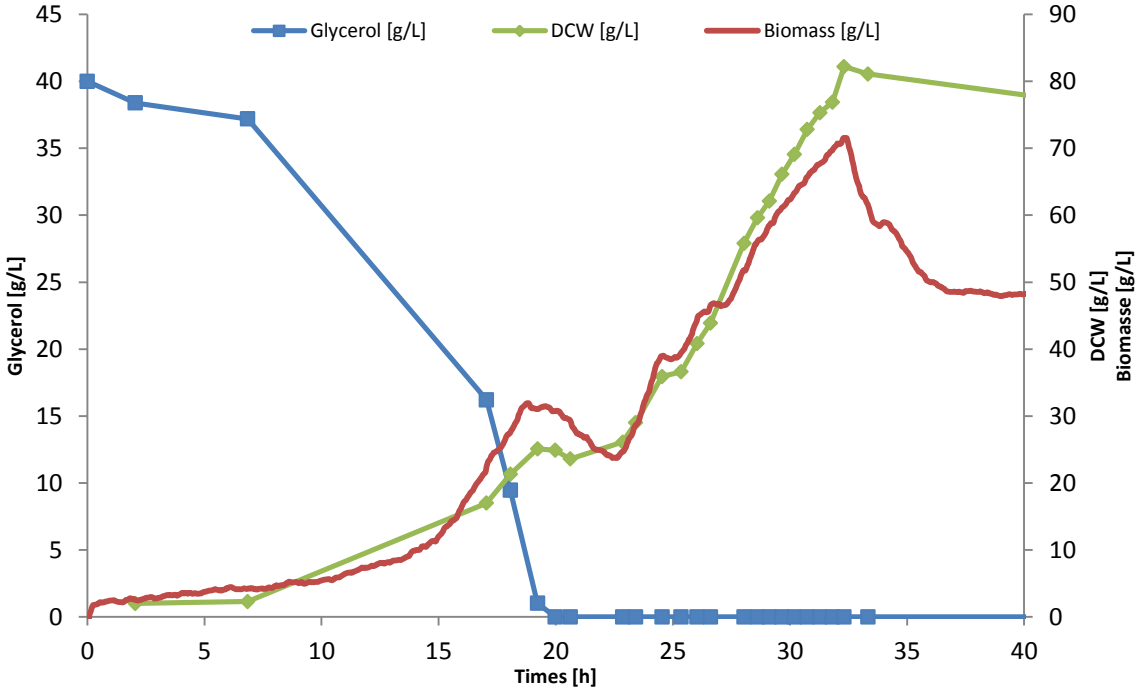


Figure 31: Substrate and biomass concentration after 40 [h] of culture

Feed glycerol was started at **22.3 [h]** with F0 = 0.425 [g/min] and μ_{sp} = 0.17 [h^{-1}]. F0 was inserted manually because the feed did not start. A comprehensive review of the program Labview would be appropriate to solve this kind of problem during fermentation. It is found that the CO_2 production restarts and different profiles of biomass and optical density can be observed. However **figure 31** shows that the concentration of **glycerol is 0 [g/L]** in the feed phase. This provides optimal conditions for biomass production (**dS/dT = 0**). Thus the implementation of feedback controller works for μ_{sp} = 0.17 [h^{-1}]. Nevertheless, the critical parameter here is the determination of µ. If the drift of biomass monitor or data reconciliation leads to the excessive fluctuation µ is wrong, as a result, the feed rate is decreased. To prevent this problem, adding a new parameter for calculating the µ would be wiser in increase the accuracy of the feed rate.

At 32.1 [h] glycerol feed is over, **biomass** is **70.7 [g/L]**, **μ_{max} = 0.154 [h^{-1}]**, **$Y_{x/s}$= 0.39 [g/g]**, **t_d = 4.50 [h]**, **r_x = 6.62 [g/L/h]**, **$-r_s$ = -17.0 [g/L/h] and q_s = 0.39 [g/g/h].** A peak is found at 33.8 [h] in the profile of CO_2. This is due to the injection of 9 g of NH_4Cl in the reactor to potentially overcome the problem due to nitrogen limitation. For the next fermentation, the amount of nitrogen will be increased. It is likely that during the filtration of the media some nutrients remain attached to the filter.

To improve the protein production and minimize the evaporation of methanol, we firstly injected methanol directly into the environment rather than on the surface, secondly the temperature was maintained at 25 °C and pO_2 at 40%. This temperature was chosen to help the production of heterologous protein **[31]**, *Pichia pastoris* produces more heat during induction and as seen in previous fermentations our cooling system does not seem to keep the temperature constant at 30 °C.

We have scrupulously followed the method of Invitrogen **[27]**. **Figure 32** shows the evolution of biomass after induction. It was found that the temperature change made at 41.7 [h], causes a signal increase in the biomass monitoring. This is normal because the polarizing effect of the probe changes with temperature and the conductivity in particular. It was noted later that the biomass monitor was off after a few hours. The **methanol feed** starts at **47.5 [h]**. There is an accumulation of methanol in the reactor. The concentration of methanol is toxic to the cell above 7.6 [g/L] **[27]**. This concentration is reached at 94 [h] and then the methanol concentration increases excessively. We can clearly see the limits of our method. Indeed, the strain is Mut^s suggesting a very low consumption of methanol. Therefore there should be absolutely a methanol monitoring and control in the reactor.

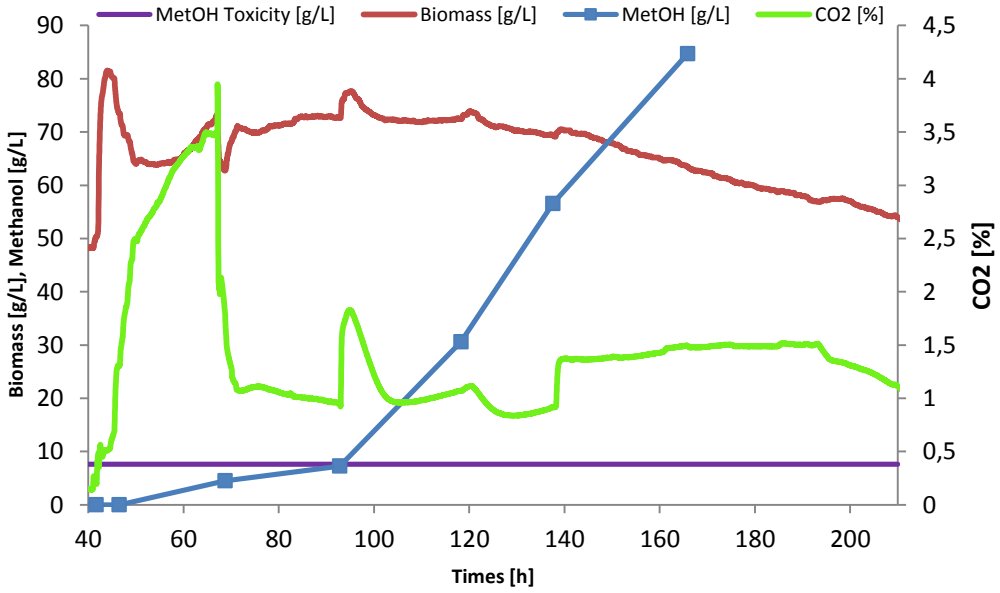


Figure 32: Evolution methanol during induction phase

After 192 [h] of culture and induction at 144.6 [h], the **concentration of HSA** is **1.13 [g/L]** (**table 11**). The hypothesis of toxicity seems to be confirmed by the concentration of methanol in the reactor. In addition we find that the concentration does not increase hSA from 137 to 192 [h]. So it is more likely that a large number of cells are dead and that they released their proteins. The **specific volumetric productivity** is **5.88 [mg hSA/L/h], the global productivity is 2 [g hSA], $Y_{P/S}$ = 0.0027 [g hSA/g methanol], $Y_{P/X}$ = 0.020 [g hSA/g biomass], $Y_{X/S}$ = -0.021 [g biomass/g methanol], q_P = 0.014 [mg/g/h].**

Tableau 11: Protein concentration during induction

Times [h]	137.58	165.88	192.05
OD_{595nm} [-]	0.499	0.495	0.510
BSA [g/L]	1.03	1.02	1.05
hSA [g/L]	1.10	1.09	1.13

3.2.FTIR

3.2.1. Model building

To improve the online monitoring and responding to the problem encountered during the various fermentations, we created three models. These models follow online glycerol, methanol, ammonium and phosphate. **Table 12** and **13** shows the standard error of calibration (SEC) from the creation of models with two different factors in the compound. It is found the SEC calibrations 07 and 08 are quite similar in contrast to calibration 06. This can be explained by the course of the experiment. Indeed Cal07 and 08 were performed on the same day. We have seen during fermentation that the probability that the lamp of FTIR is the end of life and is no longer effective as and when it is used. So it is very likely that the SEC be influenced by that.

Table 12: Output after create model using factor 6 6 6 6

Calibration	PLS Factor	SEC [g/L]			
		Glycerol	MetOH	NH_4	PO_4
Cal06	6 6 6 6	0.0824	0.238	0.0518	0.1407
Cal07	6 6 6 6	0.3933	0.5099	0.079	0.5412
Cal08	6 6 6 6	0.4723	0.4787	0.0809	0.4506

Table 13: Output after create model using factor 4 4 4 4

Calibration	PLS Factor	SEC [g/L]			
		Glycerol	MetOH	NH_4	PO4
Cal06	4 4 4 4	0.5088	0.3715	0.0569	0.2913
Cal07	4 4 4 4	0.8214	1.3291	0.0944	0.8631
Cal08	4 4 4 4	0.8183	1.2446	0.1123	0.6879

3.2.2. Protein detection

In this section we will observe different correlations between the concentrations of several proteins and intensities obtained by FTIR. The three wavelengths in the following tables are selected according to the best determination coefficients obtained for each wavelength (Annexe 5).

3.2.2.1. FTIR type N°1

It can be seen in table 14 that wavenumbers 1918, 1825 and 1605 [cm^{-1}] give the best determination coefficients for BSA. 1918 and 1825 [cm^{-1}] have coefficient greater than 0.999 between 0.625 and 10 [g/L]. 1825 [cm^{-1}] appears to be due to the cysteine of the protein [32]. 1605 [cm^{-1}], between 0625 and 10 [g/L] the coefficient is at least 0.9765. So this would correspond to an amide I [33].

Table 14: Determination coefficient for BSA

	BSA [g/L]							
Wavenumber [cm-	**0.078**	**0.156**	**0.313**	**0.625**	**1.25**	**2.5**	**5**	**10**
1918	R^2=0.8799							
		R^2=0.9136						
			R^2=0.9985					
				R^2=0.9992				
					R^2=0.9999			
1825	R^2=0.9243							
		R^2=0.9232						
			R^2=0.9930					
				R^2=0.9991				
					R^2=0.9994			
1605	R^2=0.5922							
		R^2=0.7127						
			R^2=0.7525					
				R^2=0.9803				
					R^2=0.9765			

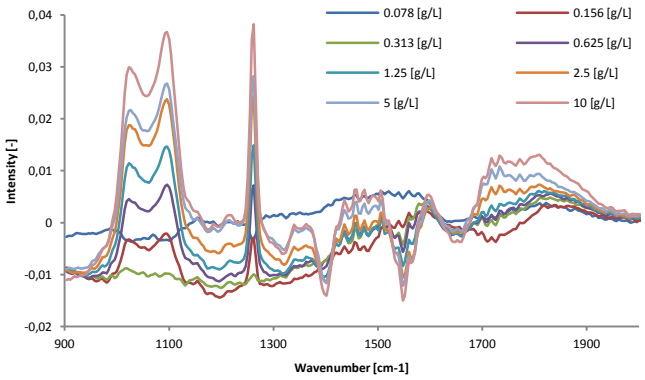


Table 15 shows the results for the hSA. To 1721 [cm^{-1}], the best coefficient are around 0.96 for a range of 0.313-10 [g/L], this wavelength could be aspartate [32]. With 1551 [cm^{-1}], the best coefficient are around 0.95 for a range of 0.313-10 [g/L] corresponding to an amide II [33]. To 1547 [cm^{-1}], the best correlations are around 0.95 for a range of 0.313-10 [g/L] are corresponding to an amide II [33].

Table 15: Determination coefficient for hSA

	hSA [g/L]							
Wavenumber [cm-	**0.078**	**0.156**	**0.313**	**0.625**	**1.25**	**2.5**	**5**	**10**
1721	R^2=0.5930							
		R^2=0.8755						
			R^2=0.9664					
				R^2=0.9731				
					R^2=0.9696			
1551	R^2=0.8905							
		R^2=0.8873						
			R^2=0.9468					
				R^2=0.9594				
					R^2=0.9580			
1547	R^2=0.8859							
		R^2=0.8839						
			R^2=0.9518					
				R^2=0.9669				
					R^2=0.9661			

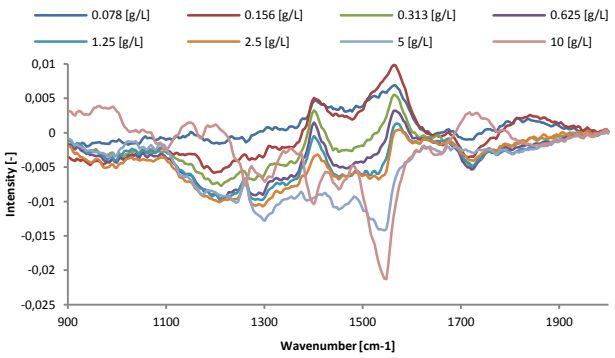
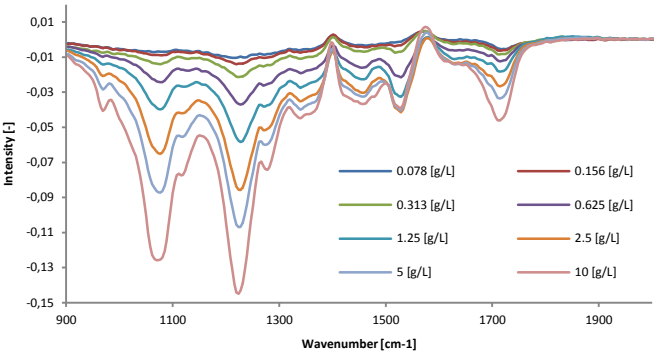


Table 16, corresponding to pepsin, the optimal coefficient 0.9714 is obtained at a wavelength of 1706 [cm^{-1}] with a range of 1.25-10 [g/L] and is an amide I [33]. In 1212 [cm^{-1}], the best result is 0.9625 with a range of 1.25-10 [g/L] corresponding to an amide III [33]. For 1011 [cm^{-1}], the best result is 0.9851 with a range of 1.25-10 [g/L], corresponding to tryptophan [32].

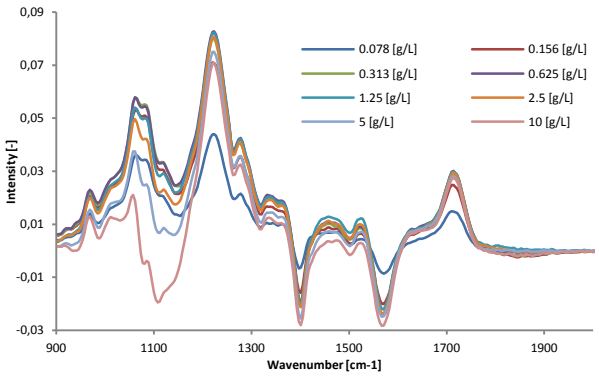
Table 16: Determination coefficient for Pepsin

	Pepsin [g/L]							
Wavenumber [cm-1]	**0.078**	**0.156**	**0.313**	**0.625**	**1.25**	**2.5**	**5**	**10**
1706	R^2=0.9257							
		R^2=0.9299						
			R^2=0.9382					
				R^2=0.9511				
					R^2=0.9714			
1212	R^2=0.9011							
		R^2=0.9043						
			R^2=0.9138					
				R^2=0.9351				
					R^2=0.9625			
1011	R^2=0.9555							
		R^2=0.9563						
			R^2=0.9640					
				R^2=0.9723				
					R^2=0.9851			



In table 17, a good correlation of the lipase. In 1628 [cm^{-1}], there is good coefficient 0.9928 between 1.25-10 [g/L] amide I [33]. For 1555 [cm^{-1}], amide II [33], a coefficient of 0.9954 for the same concentrations. However, for 1107 [cm^{-1}], we get a good coefficient between 0.156-10 [g/L], corresponding to histidine [32].

Table 17: Determination coefficient for Lipase

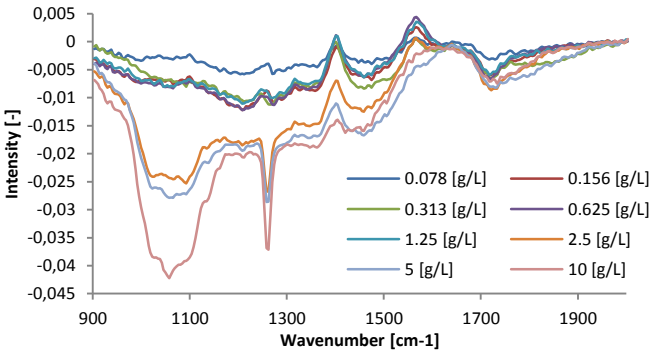


	Lipase [g/L]							
Wavenumber [cm-1]	**0.078**	**0.156**	**0.313**	**0.625**	**1.25**	**2.5**	**5**	**10**
1628	R^2=0.0018							
		R^2=0.2272						
			R^2=0.9452					
				R^2=0.9494				
					R^2=0.9928			
1555	R^2=0.4790							
		R^2=0.7866						
			R^2=0.7804					
				R^2=0.8413				
					R^2=0.9954			
1107	R^2=0.9230							
		R^2=0.9939						
			R^2=0.9989					
				R^2=0.9988				
					R^2=0.9991			

For α-Amylase, table 18, at 1640 [cm^{-1}] corresponding to amide I, we obtain a correlation 0.9306 between 1.25-10 [g/L]. To 1057 [cm^{-1}], we see that for the greater range of concentration of 0.078-10 [g/L] was one of the best correlation. However for 992 [cm^{-1}], the result is very interesting because more we considerate the range of concentration more the coefficient is better. Here 0.8953 its better coefficient between 0.078-10 [g/L], it's corresponding probably to serine [32].

Table 18: Determination coefficient for α-Amylase

	α-Amylase [g/L]							
Wavenumber [cm⁻	**0.078**	**0.156**	**0.313**	**0.625**	**1.25**	**2.5**	**5**	**10**
1640	R^2=0.4920							
		R^2=0.5022						
			R^2=0.4846					
				R^2=0.7333				
					R^2=0.9306			
1057	R^2=0.9220							
		R^2=0.9229						
			R^2=0.9135					
				R^2=0.8985				
					R^2=0.8711			
992	R^2=0.8953							
		R^2=0.8980						
			R^2=0.8906					
				R^2=0.8806				
					R^2=0.8461			



The results show characteristic peaks for proteins that do not interfere with the wavelengths of the various compounds used to develop model for monitoring of other media components by spectroscopic methods [21]. The table 19 shows the spectral range of the various compound often used in the culture medium and in particular in this work. We can see that if we can detect a protein of interest outside the spectral range of these compounds, we can monitor and control protein production on-line. This opens up very exciting prospect for PAT.

Table 19: Characteristics of the calibration model used for the FTIR [21]

Analyte	Concentration Range (g/L)	Spectral Range (cm^{-1})	PLS Factors	SEC (g/L)
Glucose	0–25	1,200-950	7	0.47
Ethanol	0–10	1,150-950	10	0.10
Ammonium	0–2	1,500-1,400 & 1,200-1,000	4	0.04
Phosphate	0–4	1,200-1,000	5	0.11
Glycerol	0–2	1,200-1,000	8	0.10
Acetic acid	0–2	1,500-950	10	0.03

Therefore, integrating information on protein levels in the models is promising. The development of a model including information on protein levels seems possible and promising.

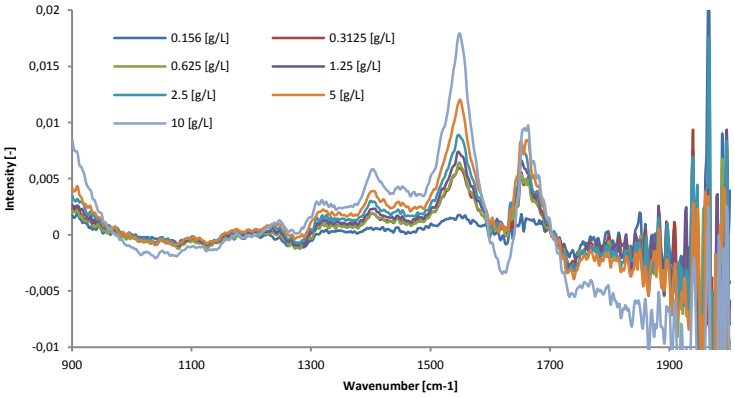
3.2.2.2. FTIR type n°2

We decided to visualize the behaviour of IR spectra using another FTIR more recently manufactured. The same method is used to select the wavelengths. In addition they are the same solutions but only the concentrations vary from 0.156-10 [g/L].

Table 20 shows the results for BSA. There is a first step the coefficients of determination were all greater than 0.9 for a range of 0.156-10 [g/L]. In 1825 [cm^{-1}], corresponding to the wavelength of absorbance of cysteine [32], the best ratio is in the range 1.25-10 [g/L]. To 1547 [cm^{-1}], between 0.313-10 [g/L], we have a very good coefficient, due to an amide II [33]. We get the same range to 1286 [cm-1] corresponding to an amide III [33].

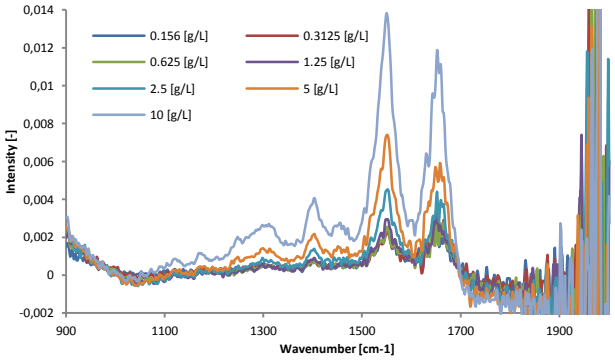
Table 20: Determination coefficient for BSA

	BSA [g/L]						
Wavenumber [cm-1]	0.156	0.313	0.625	1.25	2.5	5	10
1825	R^2=0.9659						
		R^2=0.9662					
			R^2=0.9749				
				R^2=0.9907			
1547	R^2=0.9171						
		R^2=0.9995					
			R^2=0.9998				
				R^2=1.0000			
1286	R^2=0.9690						
		R^2=0.9925					
			R^2=0.9928				
				R^2=0.9907			



The coefficients for the hSA are reported in **table 21**. Here the coefficients are better than those of BSA. They are all above 0.99 and the range of 0.156-10 [g/L], 1557 [cm-1] for an amide II [33], 1452 [cm-1] for a methyl group [32] and 1411 [cm^{-1}] for tryptophan [32].

Table 21: Determination coefficient for hSA



	hSA [g/L]						
Wavenumber [cm^{-1}]	0.156	0.313	0.625	1.25	2.5	5	10
1557	R^2=0.9951						
		R^2=0.9980					
			R^2=0.9999				
				R^2=0.9999			
1452	R^2=0.9934						
		R^2=0.9931					
			R^2=0.9975				
				R^2=1.0000			
1411	R^2=0.9965						
		R^2=0.9931					
			R^2=0.9975				
				R^2=0.9998			

Table 22 used to display the coefficients for the lipase. One can see that for 1256 [cm^{-1}], the amide III band [33], the best results are in the range 0.625-10 coefficient [g/L]. To 1094 [cm^{-1}], due to histidine [32], 0156-10 [g/L] the coefficient is above 0.999. The same findings are observed in 1025 [cm^{-1}], due to serine [32], but with a minimum coefficient of 0.99.

Table 22: Determination coefficient for Pepsin

	Pepsin [g/L]						
Wavenumber [cm^{-1}]	**0.156**	**0.313**	**0.625**	**1.25**	**2.5**	**5**	**10**
1648	R^2=0.8998						
		R^2=0.9339					
			R^2=0.9369				
				R^2=0.9855			
1587	R^2=0.9629						
		R^2=0.9721					
			R^2=0.9852				
				R^2=0.9824			
1076	R^2=0.9442						
		R^2=0.9601					
			R^2=0.9753				
				R^2=0.9856			

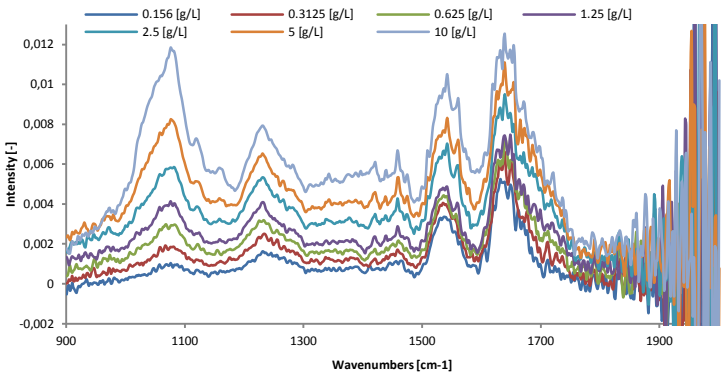
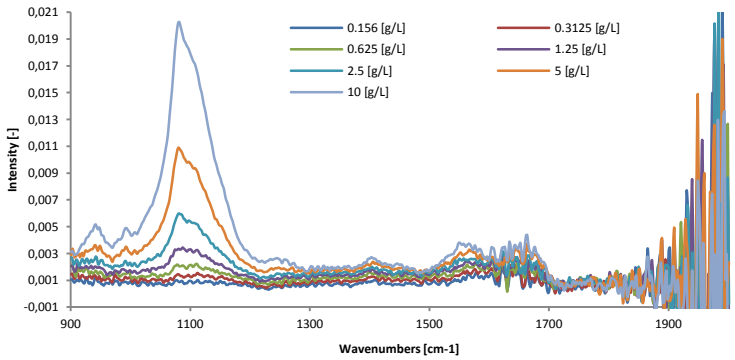


Table 23 is used to display the coefficients for the lipase. One can see that 1256 [cm^{-1}], amide III [32], that the best results in the range 0.625-10 coefficient [g/L]. To 1094 [cm^{-1}], due to histidine [33], 0156-10 [g/L] the coefficient is above 0.999. Same findings in 1025 [cm^{-1}], due to serine [33], but with a minimum coefficient of 0.99.

Table 23: Determination coefficient for Lipase

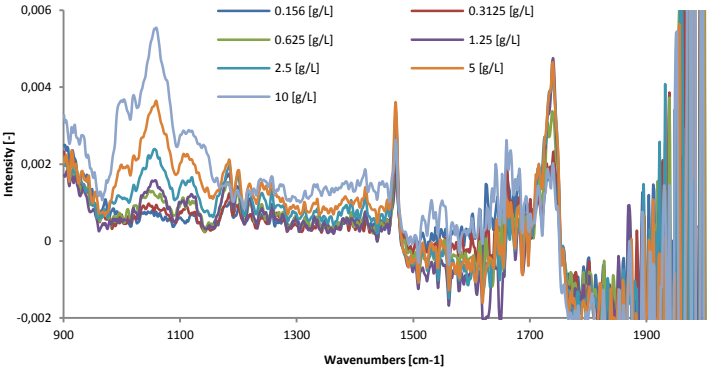
	Lipase [g/L]						
Wavenumber [cm^{-1}]	**0.156**	**0.313**	**0.625**	**1.25**	**2.5**	**5**	**10**
1256	R^2=0.9205						
		R^2=0.9567					
			R^2=0.9917				
				R^2=0.9993			
1094	R^2=0.9995						
		R^2=0.9998					
			R^2=0.9998				
				R^2=1.0000			
1025	R^2=0.9928						
		R^2=0.9961					
			R^2=0.9974				
				R^2=0.9993			



Coefficients for α-Amylase are shown in **Table 24**. To 1329 [cm^{-1}], -CH bond [33] in the 0.625-10 range [g/L] we have the best coefficient. However at 1141 [cm^{-1}], corresponding to threonine [32], the lowest coefficient is only 0.9934 for all concentrations. Also to 1018 [cm^{-1}], due to tryptophan [33], the coefficients are greater than 0.99 in the 0.156-10 range [g/L].

Table 24: Determination coefficient for α-Amylase

	α-Amylase [g/L]						
Wavenumber [cm-1]	**0.156**	**0.313**	**0.625**	**1.25**	**2.5**	**5**	**10**
1329	R^2=0.9656						
		R^2=0.9703					
			R^2=0.9999				
				R^2=0.9999			
1141	R^2=0.9934						
		R^2=0.9970					
			R^2=0.9980				
				R^2=0.9974			
1018	R^2=0.9980						
		R^2=0.9979					
			R^2=0.9975				
				R^2=0.9982			



So we can see the usefulness of his master devices and regularly checked the condition of the tools used. However we note that the results allow identifying and quantifying the five proteins studied.

4. Overview and Perspectives

Table 25 shows the overview results of fermentation carried out in this work.

Table 25: Overview of result

		Batch phase				Feed phase				Induction phase (MetOH)	
Strain	Fermentation	Glycerol [g/L]	Biomass [g/L]	Yx/s [g/g]	µmax [h-1]	Glycerol [g/L]	Biomass [g/L]	Yx/s [g/g]	µmax [h-1]	hSA [g/L]	hSA [mg/L/h]
	PpasFB01	20	no growth								
1	PpasFB02	10	6.3	0.64	0.26	300	33	0.48	0.22	0	0
	PpasFB03	10	NA	NA	NA	500	50	NA	NA	0	0
	PpasFB04	10	2.4	0.24		500	62	0.41	0.12	0.27	0.27
2	PpasFB05	40	31.6	0.8	0.15	500	76	0.41	0.15	0.11	0.11
	PpasFB06	40	29.3	0.75	0.19	500	76.6	0.82	0.15	0.01	0
3	PpasFB07	40	31.8	0.78	0.18	500	70.7	0.39	0.15	1.1	6.6

We could notice the presence of turbidity when adding the solution PTM1. To reduce the cloudyness of the fluid, it would be interesting to replace the 13.35 mL of phosphoric acid by 25 g sodium hexametaphosphate in the BSM medium [34].

It would be wise to improve the data acquisition of data and and including data reconciliation systems. To this end MatLab and LabView coding should be updated. For this update the software MatLab and LabView and a new, more reliable function for the addition of glycerol feed should be developed. Implemented new function more reliable at the launch of glycerol feed. Indeed, the establishment of a new method to determine specific growth of rate is very interesting. The data reconciliation can thus compare the two methods and choose the most reliable calculations.

To add more options to the data analysis, the Bioengineering process parameter such as pO2, temperature, pH and agitation should be monitored in LabView.

To obtain more information during the process should be followed on-line using LabView pO2, temperature, pH and agitation. We have seen that the pO2% with a set point of 40% during induction was not stable. For that it would be useful to regulate the amount of oxygen entering the reactor and agitation. This could be promoting the production of hSA because methanol would be used as a source inducer.

The installation of a reliable and accurate method would be useful for identification and quantification of hSA during the process. Although the SDS-PAGE is a method off-line, it would be very convenient to establish initially the actual output and a comparison with the FTIR.

This work shows that we could also show that the FTIR can be used to monitor the concentration and of metabolite and detect hSA. However, it appears that the light source unit is defective after hours of use. It is important to change the source and repeat the model to improve accuracy in predictions of concentrations. Furthermore, results for the proteins are promising for the quantification on-line. In this the first time analysis will be done initially in media to visualize the effect of metabolites on protein detection. Then in the media containing cells to the possible effect of observe release proteins by the cells died.

5. Conclusion

The use of methylotrophic yeasts becomes increasingly current in industry and using *Pichia pastoris* as a host for heterologous protein expression as well. We developed a simple fed-batch strategy for growing *Pichia pastoris*. Our study can answer the first objective that was the set-up of the process and we have a HCDC of 76 [g/L]. However, we have established the strain was not potentially producing hSA for genetic reasons.

We also established that the detection and quantification of protein in water, and namely hSA, was possible by FTIR. More results from this work are promising and can be considered for on-line monitoring during production of heterologous protein.

6. Acknowledgments

I thank God, the Merciful, the Just, otherwise I would not have succeeded and for allowing me to reach my current level of study and for giving me the opportunity to realize my diploma work in Ireland.

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Thanks

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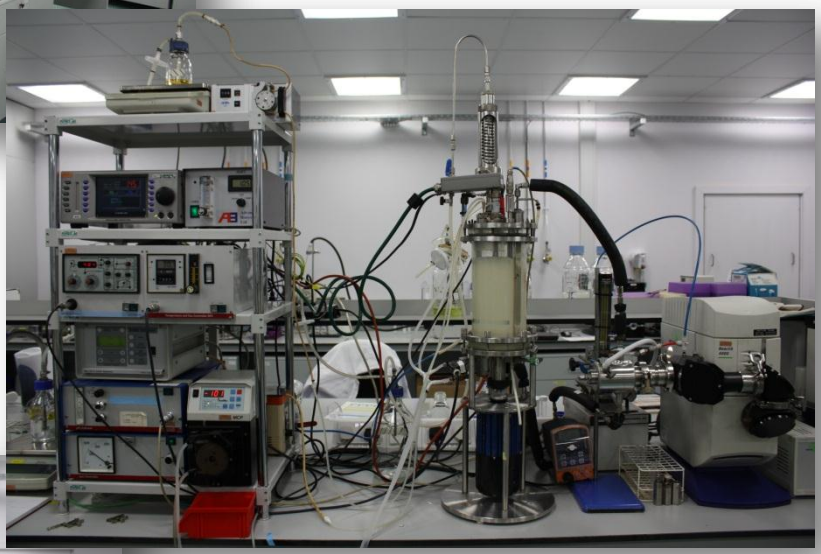
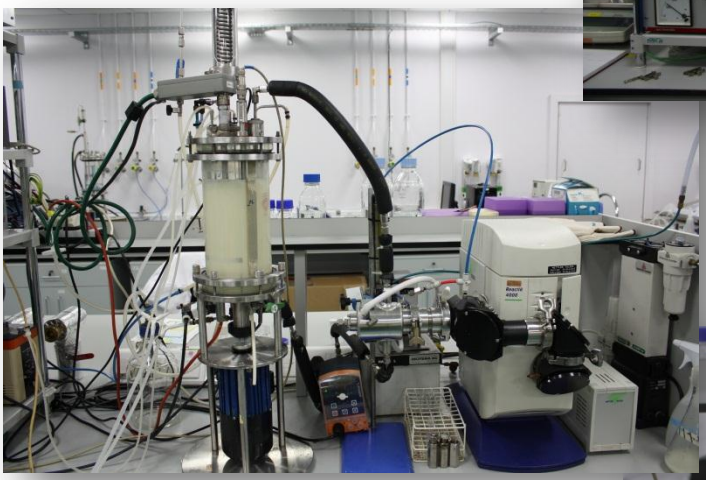
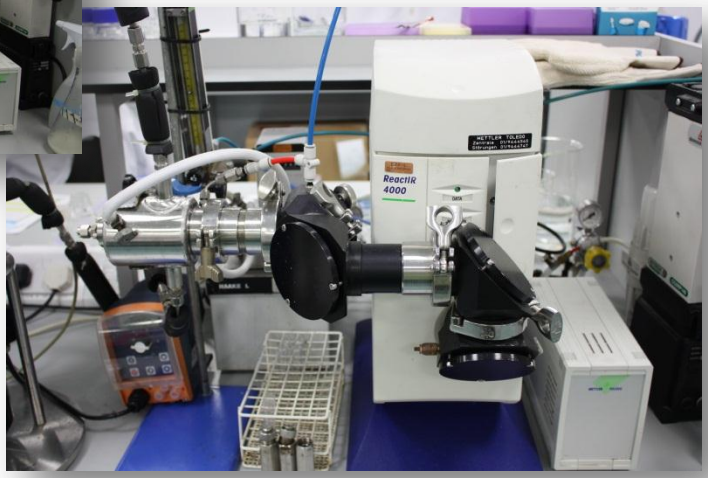
8. Annexes

8.1.Annexe 1: Reactor







8.2.Annexe 2: Matrix for 6 species 7 levels experimental design

MATRIX FOR 6-SPECIES 7-LEVEL EXPERIMENTAL DESIGN - *ReactIR3 FTIR* - 26.03.2010 - MD/MMS

Std #	Glucose	EtOH	NH4	PO4	Glycerol	AcAc	BUFFER	Std #	Glucose	EtOH	(NH4)2SO4	KH2PO4	Glycerol	AcAc	Glucose	EtOH	NH4	PO4	Glycerol	AcAc	Std #
1	3	3	3	3	3	3	9	1	5.00	2.00	3.66	2.87	1.00	1.00	5.00	2.00	1.00	2.00	1.00	1.00	1
2	**3**	0	1	2	2	5	14	2	5.00	0.00	1.22	1.91	0.67	1.67	5.00	0.00	0.33	1.33	0.67	1.67	2
3	0	1	2	2	5	4	13	3	0.00	0.67	2.44	1.91	1.67	1.33	0.00	0.67	0.67	1.33	1.67	1.33	3
4	1	2	2	5	4	5	8	4	1.67	1.33	2.44	4.78	1.33	1.67	1.67	1.33	0.67	3.33	1.33	1.67	4
5	2	2	5	4	5	3	6	5	3.33	1.33	6.10	3.82	1.67	1.00	3.33	1.33	1.67	2.67	1.67	1.00	5
6	2	5	4	5	3	4	4	6	3.33	3.33	4.88	4.78	1.00	1.33	3.33	3.33	1.33	3.33	1.00	1.33	6
7	5	4	5	3	4	0	6	7	8.33	2.67	6.10	2.87	1.33	0.00	8.33	2.67	1.67	2.00	1.33	0.00	7
8	4	5	3	4	0	5	6	8	6.67	3.33	3.66	3.82	0.00	1.67	6.67	3.33	1.00	2.67	0.00	1.67	8
9	5	3	4	0	5	5	5	9	8.33	2.00	4.88	0.00	1.67	1.67	8.33	2.00	1.33	0.00	1.67	1.67	9
10	**3**	4	0	5	5	6	4	10	5.00	2.67	0.00	4.78	1.67	2.00	5.00	2.67	0.00	3.33	1.67	2.00	10
11	4	0	5	5	6	2	5	11	6.67	0.00	6.10	4.78	2.00	0.67	6.67	0.00	1.67	3.33	2.00	0.67	11
12	0	5	5	6	2	6	3	12	0.00	3.33	6.10	5.73	0.67	2.00	0.00	3.33	1.67	4.00	0.67	2.00	12
13	5	5	6	2	6	3	0	13	8.33	3.33	7.32	1.91	2.00	1.00	8.33	3.33	2.00	1.33	2.00	1.00	13
14	5	6	2	6	3	2	3	14	8.33	4.00	2.44	5.73	1.00	0.67	8.33	4.00	0.67	4.00	1.00	0.67	14
15	6	2	6	3	2	4	4	15	10.00	1.33	7.32	2.87	0.67	1.33	10.00	1.33	2.00	2.00	0.67	1.33	15
16	2	6	3	2	4	6	4	16	3.33	4.00	3.66	1.91	1.33	2.00	3.33	4.00	1.00	1.33	1.33	2.00	16
17	6	3	2	4	6	6	0	17	10.00	2.00	2.44	3.82	2.00	2.00	10.00	2.00	0.67	2.67	2.00	2.00	17
18	**3**	2	4	6	6	1	5	18	5.00	1.33	4.88	5.73	2.00	0.33	5.00	1.33	1.33	4.00	2.00	0.33	18
19	2	4	6	6	1	5	3	19	3.33	2.67	7.32	5.73	0.33	1.67	3.33	2.67	2.00	4.00	0.33	1.67	19
20	4	6	6	1	5	1	4	20	6.67	4.00	7.32	0.96	1.67	0.33	6.67	4.00	2.00	0.67	1.67	0.33	20
21	6	6	1	5	1	3	5	21	10.00	4.00	1.22	4.78	0.33	1.00	10.00	4.00	0.33	3.33	0.33	1.00	21
22	6	1	5	1	3	5	6	22	10.00	0.67	6.10	0.96	1.00	1.67	10.00	0.67	1.67	0.67	1.00	1.67	22
23	1	5	1	3	5	2	10	23	1.67	3.33	1.22	2.87	1.67	0.67	1.67	3.33	0.33	2.00	1.67	0.67	23
24	5	1	3	5	2	1	10	24	8.33	0.67	3.66	4.78	0.67	0.33	8.33	0.67	1.00	3.33	0.67	0.33	24
25	1	3	5	2	1	1	14	25	1.67	2.00	6.10	1.91	0.33	0.33	1.67	2.00	1.67	1.33	0.33	0.33	25
26	**3**	5	2	1	1	0	15	26	5.00	3.33	2.44	0.96	0.33	0.00	5.00	3.33	0.67	0.67	0.33	0.00	26
27	5	2	1	1	0	6	12	27	8.33	1.33	1.22	0.96	0.00	2.00	8.33	1.33	0.33	0.67	0.00	2.00	27
28	2	1	1	0	6	0	17	28	3.33	0.67	1.22	0.00	2.00	0.00	3.33	0.67	0.33	0.00	2.00	0.00	28
29	1	1	0	6	0	3	16	29	1.67	0.67	0.00	5.73	0.00	1.00	1.67	0.67	0.00	4.00	0.00	1.00	29
30	1	0	6	0	3	6	11	30	1.67	0.00	7.32	0.00	1.00	2.00	1.67	0.00	2.00	0.00	1.00	2.00	30
31	0	6	0	3	6	5	7	31	0.00	4.00	0.00	2.87	2.00	1.67	0.00	4.00	0.00	2.00	2.00	1.67	31
32	6	0	3	6	5	0	7	32	10.00	0.00	3.66	5.73	1.67	0.00	10.00	0.00	1.00	4.00	1.67	0.00	32
33	0	3	6	5	0	0	13	33	0.00	2.00	7.32	4.78	0.00	0.00	0.00	2.00	2.00	3.33	0.00	0.00	33
34	**3**	6	5	0	0	4	9	34	5.00	4.00	6.10	0.00	0.00	1.33	5.00	4.00	1.67	0.00	0.00	1.33	34
35	6	5	0	0	4	1	11	35	10.00	3.33	0.00	0.00	1.33	0.33	10.00	3.33	0.00	0.00	1.33	0.33	35
36	5	0	0	4	1	4	13	36	8.33	0.00	0.00	3.82	0.33	1.33	8.33	0.00	0.00	2.67	0.33	1.33	36
37	0	0	4	1	4	3	15	37	0.00	0.00	4.88	0.96	1.33	1.00	0.00	0.00	1.33	0.67	1.33	1.00	37
38	0	4	1	4	3	1	14	38	0.00	2.67	1.22	3.82	1.00	0.33	0.00	2.67	0.33	2.67	1.00	0.33	38
39	4	1	4	3	1	6	8	39	6.67	0.67	4.88	2.87	0.33	2.00	6.67	0.67	1.33	2.00	0.33	2.00	39
40	1	4	3	1	6	4	8	40	1.67	2.67	3.66	0.96	2.00	1.33	1.67	2.67	1.00	0.67	2.00	1.33	40
41	4	3	1	6	4	4	5	41	6.67	2.00	1.22	5.73	1.33	1.33	6.67	2.00	0.33	4.00	1.33	1.33	41
42	**3**	1	6	4	4	2	7	42	5.00	0.67	7.32	3.82	1.33	0.67	5.00	0.67	2.00	2.67	1.33	0.67	42
43	1	6	4	4	2	0	10	43	1.67	4.00	4.88	3.82	0.67	0.00	1.67	4.00	1.33	2.67	0.67	0.00	43
44	6	4	4	2	0	2	9	44	10.00	2.67	4.88	1.91	0.00	0.67	10.00	2.67	1.33	1.33	0.00	0.67	44
45	4	4	2	0	2	3	12	45	6.67	2.67	2.44	0.00	0.67	1.00	6.67	2.67	0.67	0.00	0.67	1.00	45
46	4	2	0	2	3	0	16	46	6.67	1.33	0.00	1.91	1.00	0.00	6.67	1.33	0.00	1.33	1.00	0.00	46
47	2	0	2	3	0	1	19	47	3.33	0.00	2.44	2.87	0.00	0.33	3.33	0.00	0.67	2.00	0.00	0.33	47
48	0	2	3	0	1	2	19	48	0.00	1.33	3.66	0.00	0.33	0.67	0.00	1.33	1.00	0.00	0.33	0.67	48
49	2	3	0	1	2	2	17	49	3.33	2.00	0.00	0.96	0.67	0.67	3.33	2.00	0.00	0.67	0.67	0.67	49
50	0	0	0	0	0	0	27	50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	50
									g/l						g/l						

Bulk soln	g/l	g / 100 ml
Glucose	45	4.500
EtOH	18	1.800
(NH4)2SO4	32.9360111	3.294
KH2PO4	25.793619	2.579
Glycerol	9	0.900
AcAc	9	0.900

1 unit = 10/24 = 0.4166 ml
27 units each standard
11.25 ml each standard
147 units of each product
147 * 10 / 24 = 61 mL

pH = 5
T = 30°C

8.3.Annexe 3: Matrix for 4 species 7 levels experimental design

MATRIX FOR 4-SPECIES 7-LEVEL EXPERIMENTAL DESIGN - *ReactIR3 FTIR*

Std #	Glycerol	Methanol	NH4	PO4	BUFFER	Std #	Glycerol	Methanol	NH4Cl	KH2PO4	Glycerol	Methanol	NH4	PO4	Std #
1	3	3	3	3	7	1	5.00	5.01	5.92	6.19	5.00	5.01	2.00	6.00	1
2	**3**	0	1	2	13	2	5.00	0.00	1.97	4.13	5.00	0.00	0.67	4.00	2
3	0	1	2	2	14	3	0.00	1.67	3.95	4.13	0.00	1.67	1.33	4.00	3
4	1	2	2	5	9	4	1.67	3.34	3.95	10.32	1.67	3.34	1.33	10.00	4
5	2	2	5	4	6	5	3.34	3.34	9.87	8.26	3.34	3.34	3.33	8.00	5
6	2	5	4	5	3	6	3.34	8.36	7.89	10.32	3.34	8.36	2.66	10.00	6
7	5	4	5	3	2	7	8.34	6.68	9.87	6.19	8.34	6.68	3.33	6.00	7
8	4	5	3	4	3	8	6.67	8.36	5.92	8.26	6.67	8.36	2.00	8.00	8
9	5	3	4	0	7	9	8.34	5.01	7.89	0.00	8.34	5.01	2.66	0.00	9
10	**3**	4	0	5	7	10	5.00	6.68	0.00	10.32	5.00	6.68	0.00	10.00	10
11	4	0	5	5	5	11	6.67	0.00	9.87	10.32	6.67	0.00	3.33	10.00	11
12	0	5	5	6	3	12	0.00	8.36	9.87	12.39	0.00	8.36	3.33	12.00	12
13	5	5	6	2	1	13	8.34	8.36	11.84	4.13	8.34	8.36	4.00	4.00	13
14	5	6	2	6	0	14	8.34	10.03	3.95	12.39	8.34	10.03	1.33	12.00	14
15	6	2	6	3	2	15	10.01	3.34	11.84	6.19	10.01	3.34	4.00	6.00	15
16	2	6	3	2	6	16	3.34	10.03	5.92	4.13	3.34	10.03	2.00	4.00	16
17	6	3	2	4	4	17	10.01	5.01	3.95	8.26	10.01	5.01	1.33	8.00	17
18	**3**	2	4	6	4	18	5.00	3.34	7.89	12.39	5.00	3.34	2.66	12.00	18
19	2	4	6	6	1	19	3.34	6.68	11.84	12.39	3.34	6.68	4.00	12.00	19
20	4	6	6	1	2	20	6.67	10.03	11.84	2.06	6.67	10.03	4.00	2.00	20
21	6	6	1	5	1	21	10.01	10.03	1.97	10.32	10.01	10.03	0.67	10.00	21
22	6	1	5	1	6	22	10.01	1.67	9.87	2.06	10.01	1.67	3.33	2.00	22
23	1	5	1	3	9	23	1.67	8.36	1.97	6.19	1.67	8.36	0.67	6.00	23
24	5	1	3	5	5	24	8.34	1.67	5.92	10.32	8.34	1.67	2.00	10.00	24
25	1	3	5	2	8	25	1.67	5.01	9.87	4.13	1.67	5.01	3.33	4.00	25
26	**3**	5	2	1	8	26	5.00	8.36	3.95	2.06	5.00	8.36	1.33	2.00	26
27	5	2	1	1	10	27	8.34	3.34	1.97	2.06	8.34	3.34	0.67	2.00	27
28	2	1	1	0	15	28	3.34	1.67	1.97	0.00	3.34	1.67	0.67	0.00	28
29	1	1	0	6	11	29	1.67	1.67	0.00	12.39	1.67	1.67	0.00	12.00	29
30	1	0	6	0	12	30	1.67	0.00	11.84	0.00	1.67	0.00	4.00	0.00	30
31	0	6	0	3	10	31	0.00	10.03	0.00	6.19	0.00	10.03	0.00	6.00	31
32	6	0	3	6	4	32	10.01	0.00	5.92	12.39	10.01	0.00	2.00	12.00	32
33	0	3	6	5	5	33	0.00	5.01	11.84	10.32	0.00	5.01	4.00	10.00	33
34	**3**	6	5	0	5	34	5.00	10.03	9.87	0.00	5.00	10.03	3.33	0.00	34
35	6	5	0	0	8	35	10.01	8.36	0.00	0.00	10.01	8.36	0.00	0.00	35
36	5	0	0	4	10	36	8.34	0.00	0.00	8.26	8.34	0.00	0.00	8.00	36
37	0	0	4	1	14	37	0.00	0.00	7.89	2.06	0.00	0.00	2.66	2.00	37
38	0	4	1	4	10	38	0.00	6.68	1.97	8.26	0.00	6.68	0.67	8.00	38
39	4	1	4	3	7	39	6.67	1.67	7.89	6.19	6.67	1.67	2.66	6.00	39
40	1	4	3	1	10	40	1.67	6.68	5.92	2.06	1.67	6.68	2.00	2.00	40
41	4	3	1	6	5	41	6.67	5.01	1.97	12.39	6.67	5.01	0.67	12.00	41
42	**3**	1	6	4	5	42	5.00	1.67	11.84	8.26	5.00	1.67	4.00	8.00	42
43	1	6	4	4	4	43	1.67	10.03	7.89	8.26	1.67	10.03	2.66	8.00	43
44	6	4	4	2	3	44	10.01	6.68	7.89	4.13	10.01	6.68	2.66	4.00	44
45	4	4	2	0	9	45	6.67	6.68	3.95	0.00	6.67	6.68	1.33	0.00	45
46	4	2	0	2	11	46	6.67	3.34	0.00	4.13	6.67	3.34	0.00	4.00	46
47	2	0	2	3	12	47	3.34	0.00	3.95	6.19	3.34	0.00	1.33	6.00	47
48	0	2	3	0	14	48	0.00	3.34	5.92	0.00	0.00	3.34	2.00	0.00	48
49	2	3	0	1	13	49	3.34	5.01	0.00	2.06	3.34	5.01	0.00	2.00	49
50	0	0	0	0	19	50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	50

Bulk soln	g/l	g / 100 ml
Glycerol	31.695	**3.170**
MetOH	31.75	**3.175**
NH4Cl	37.5	**3.750**
H3PO4	39.22	**3.922**

Units =	12	(pos.4)	T	30
1 unit =	0.41666667	ml	pH	5
Each std =	7.91666667	ml	pH not adjusted during calib	
Each comp :	147	units		
Each comp :	61.25	ml		

Component	Experimental range		Glycerol	Calibration range	
	High	Low		High	Low
Glycerol	10	0	Glycerol	10	0.5
Methanol	10	0	Methanol	10	0.5
NH4Cl	9	0	NH4	4	0.5
H3PO4	14	0	PO4	12	

Prepare 125 ml of each bulk solution

Each solution	122.5	ml

8.4.Annexe 4: Media Egli and solution vitamine

Milieu Egli, Concentrations doubles Concentration minimale de biotine

Pichia pastoris, methanol selon référence 20487

			Feed (MD)
Components	**for 1L**	**units**	**for 1L**
Glycerol	20	g	300
NH_4Cl	15.26	g	91.56
KH_2PO_4	5.62	g	33.72
$MgSO_4\ 7H_2O$	1.18	g	7.08
$CaCl_2\ 2H_2O$	110	mg	660
$FeCl_3\ 6H_2O$	75	mg	450
$MnSO_4\ H_2O$	28	mg	168
$ZnSO_4\ 7H_2O$	44	mg	264
$CuSO_4\ 5H_2O$	8	mg	48
$CoCl_2\ 6\ H_2O$	8	mg	48
$Na_2MoO_4\ 2H_2O$	5.2	mg	31.2
H_3BO_3	8	mg	48
KI	1.2	mg	7.2
Biotin	0.020	mg	
PPG	0.4	mL	

Selon Carmen Jungo: "test biotin minimal". Remplacé MeOH par glycerol; struktol SB 2121 par PPG.
Feed: 15x glycerol, 6x other components

Vitamins solution	**Component**	**Concentration [mg/L]**
Sterilised at 121°C for 20 min	**$CaCl_2$ x $2H_2O$**	10
	H_3BO_3	2.67
	$CuSO_4$ x $2H_2O$	0.8
	KI	0.27
	$MnCl_2$	2.67
	Na_2MoO_4 x $2H_2O$	1.07
	$ZnSO_4$ x $7H_2O$	12
	EDTA	40
	$CoCl_2$	0.8
	$FeSO_4$ x $7H_2O$	8
Sterilised with filter-sterilised at room temperature	**Ca Pentothenate**	2.67
	Biotine	0.13
	Myo-Inositol	66.67
	Nicotinic Acid	2.67
	Para-Amino Benyoic Acid (PABA)	0.53
	Pyrodixine Hydrochloride	2.67
	Thiamine Hydrochloride	2.67

8.5.Annexe 5: Data raw

The data raw are on V-drive in Laboratory of Integrated Bioprocessing.